MUTATION STUDIES WITH BACILLUS SUBTILIS

by

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S U M M A R Y :

1. The derivation of mutational systems for Bacillus subtilis is described, following demonstration of the general unsuitability of a number of preexistent stocks for this purpose. Auxotrophs newly induced with UV, 2AP, EMS, and NTG were phenotypically classified by specific requirement and leakiness. There was no suggestion of any specificities of phenotype induced by any mutagen, although the proportion of leaky mutants varied with the mutagen. A single cycle of selective technique for auxotrophs was shown to be inefficient with the low mutagen doses used, thus stressing the desirability of repeating such cycles. Auxotrophs were examined for spontaneous and EMS-induced reversion in spot-tests, and suitable markers chosen for mutation studies. Efficiency of spot-tests has been compared with more quantitative estimates of revertibility.

2. Using these systems, secondary effects on mutation induction were studied. Two of these effects have been studied in detail. As no true reversion has yet been proved in B. subtilis, the majority of these experiments were undertaken with definite suppressor mutations.

Enhancement of mutation yields by incorporation into selective agars of traces of previous growth requirements are described with EMS and NTG, and shown to be dose dependent. Other agents were not sufficiently mutagenic to allow detection of such effects. An enhancement of mutation yield by an amino acid pool was also shown, the

"broth effect", and with all mutagens tested. For the mutagen predominantly studied, EMS, this broth effect was addition to the effect of previous growth requirements. Dose dependence of broth effects are described, differing also by the mutagen. Possibly related effects for transformation and transduction are reported.

Mutation frequency decline (MFD) occurred in an absence or insufficiency of broth. The importance of the relative proportions of cells and broth was shown for MFD prevention. MFD occurred for a number of markers which were reverted at high frequency by EMS, but suppressor specificity could not be shown due to the unavailability of true revertants. As far as possible, it has been excluded that MFD was due to mutagen-induced osmotic instability, participation of an agar component with broth, residual mutagen, or liquid holding recovery. There was no evidence of an after-effect on EMS-induced supersuppressor reversion. Leucine and uracil of the previous growth requirements were shown to reduce MFD, although only in liquid medium. Unlike the broth effect, this sparing of MFD was not dependent on the relative concentrations of cells and nutrients. The MFD-sparing was shown to be a strain-characteristic as other markers were similarly spared only in a strain UV2 background.

MFD of supersuppressor reversion was also shown after NTG-induction, in contrast to results of other workers. Survival after NTG treatment was medium-dependent, unlike

that after EMS.

Attempted mutagen interaction experiments with 2AP and UV or DEB and UV proved unsuccessful. Supersuppression was verified in two ways: by demonstrating that the suppressor could give joint relief at high frequency of the two susceptible markers when introduced by transformation or transduction, and by the successful reisolation of the original auxotrophies from a joint revertant.

3. An effect of genetic background and medium on the reversion of the his-A1 marker was detected. Addition of a threo$^-$ marker to strain UV2 produced an apparent "gene-controlled mutational stability" of the his-A1 marker.

Subsequent analysis showed that this apparent inhibition of histidine reversion was due to an interaction, not previously reported, between the threo$^-$ marker and the medium. Inhibition was found to be mediated through the exhaustion of medial threonine by residual growth of the auxotrophic background, with discrimination against histidine revertants, the great majority of which only arose during residual growth. It was suggested that revertants of other markers which arise late on plates may be similarly inhibited by a threonineless background.

These results are discussed in relation to those of other workers with other mutagens and organisms. The advantages of Bacillus subtilis are stressed - its transformability, and the ancillary effects on mutation induction with alkylating agents, which are apparently only found (to date) with this organism.

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ABBREVIATIONS

2AP	2-aminopurine.
DES	diethyl sulphate.
EMS	ethyl methanesulphonate.
EO	ethylene oxide.
HN_2	nitrogen mustard.
NMU	nitroso-methyl-urethane.
4NQO	4-nitroquinoline-1-oxide.
NTG	N-nitro-N'-methyl-N-nitrosoguanidine.
UV	ultraviolet irradiation.
LHR	liquid holding recovery.
MFD	mutation frequency decline.
PR	photoreactivation.

Abbreviations for media are to be found alongside their description in MATERIALS AND METHODS.

1. GENERAL INTRODUCTION

Mutant microorganisms have been used for diverse genetic, mutagenic, morphological, biochemical, developmental, industrial, and medical investigations. Already the use of mutant strains has provided the investigator with the means to decipher the genetic code, map genomes, study enzymatic activities, analyse biochemical pathways, together with such developmental processes as bacterial sporulation, and achieve a partial understanding of their control, render comprehensible the replication, transcription, and translation of the genetic message, begin the biochemical analysis of the phenomenon of genetic recombination, elucidate the cellular mechanisms of repair of physically or chemically induced damage to the integrity of the genome, and obtain much information about the genetics and physiology of bacteriophage infection and lysogeny. Of more immediate social value, microbial mechanisms of pathogenesis and resistance to chemotherapeutic agents are now under investigation with the same genetical techniques that have already yielded this rich harvest of biological information, and mutant strains are now commonly used in industry for the production of many bio-and organic chemicals of nutritional and other economic values. "All this to illustrate that when you have mutants you are far better off than when you don't" quoth Salvatore Luria (quoted by Schaeffer 1969). Verbum sapienti!

With the exception of many of the experiments leading to the deciphering of the genetic code, it is a truism that

all these results have been obtained from studies on small numbers of mutants with desired alterations in phenotype. The isolation of mutants which have gained or increased some property with respect to wild type is in general a simple matter of selection, but that of mutants whose interest to the investigator lies in the reduction of some cellular activity, often in toto, is a matter of difficulty, calling for ancillary enrichment techniques. It is certain that the paucity of mutants available for many of the sequential steps of cellular processes is due to inadequate recovery technique, allied to the low frequency of induction of many types of mutation. Even the enrichment techniques used to recover these precious mutants may by their very drastic action discriminate against certain phenotypes and yield an unrepresentative picture of the relative frequency of induction of many mutant types (MacDonald and Pontecorvo 1953; Woodward, de Zeeuw, and Srb 1954). It is evident that a selective technique depending for its success upon an activity performed by unmutated cells and its simultaneous absence in the required mutants may well impose a premium on the recovery of mutants totally unable to perform that activity. By this means, such biologically interesting mutants as the leaky, incompletely blocked bradytrophs and mutants of reduced (though not completely abolished) enzymatic activity may be excluded.

The problem obviously cannot be resolved by a direct selection of particular microbial auxotrophs, the only case coming to mind being the isolation from complete medium of

inositol auxotrophs of Ophiostoma following phleomycin treatment (Zetterberg - unpublished). It has also been demonstrated that selection for mutants resistant to some bacteriophages may return a high yield of particular auxotrophs. Try$^-$ and pro$^-$ mutants occur with high frequency in phage T1-resistant Escherichia coli K12 strains (Franklin, Dove, and Yanofsky 1965; Curtiss 1965), and a similar association of gal U$^-$ mutants and phage P1 resistance has recently been reported (Franklin 1969). Mutants of this type appear however to be associated with chromosomal aberrations and deletions of the parent strains. An example of a selection imposed by previous mutation has been shown by Boyer, Englesberg, and Weinberg (1962). Ara-D mutants of E. coli B/r are inhibited by arabinose, although they grow on other carbon sources. Mutants isolated from these ara-D mutants as arabinose-insensitive proved to have further mutations in ara-A, -B, or -C genes. Some mutations may demonstrate pleiotropic effects which may be valuable in their recovery. Ephrussi-Taylor (e.g. 1966) has shown that aminopterin-sensitivity in Pneumococcus may be selected by resistance to the inhibitory effects of an imbalance of such amino acids as isoleucine, leucine, and valine. Special medial additives may select mutants after they have been formed, and for this purpose aminopterin has been used for recovery of thymine-requiring mutants of Escherichia coli (Okada, Yanagisawa, and Ryan 1960, 1961), similarly trimethoprim (Stacey and Simson 1965), and of Bacillus subtilis (Farmer and Rothman 1965). Spontaneously-arising proline-

requiring auxotrophs of E. coli have likewise been specifically selected with 4-nitropyridine-1-oxide (Hirota, Inuzuka, and Tomoeda, 1966), all the mutants recovered demonstrating the same enzymatic lesion. The techniques of selective and enrichment culture have been reviewed recently by Schlegel and Jannasch (1967).

The advent of highly efficient mutagens has in recent years so increased yields of bacterial auxotrophs that several percent, even tens of percent, of colonies grown from cells surviving mutagen treatment may be found to be nutritionally deficient (Adelberg, Mandel, and Chein Ching Chen 1965, Eisenstark, Eisenstark, and van Sickle 1965). The alkylating agents EMS, and, in particular, NTG, are such mutagens which may under given experimental conditions also return very little lethality of treated cells. Adelberg et al. (1965) have calculated that an auxotrophic yield of 10% of cells surviving a NTG treatment may represent one randomly located mutation per cell. This figure is often obtained and frequently exceeded. A complication of such high mutagenicity has been the demonstration that a large proportion of the mutants induced by these agents are possessed of multiple genetic lesions (Adelberg et al. 1965, Eisenstark et al. 1965, Lindegren, Hwang, Oshima, and Lindegren 1965, Hirota, Jacob, Ryter, Buttin, and Nakai 1968). A rigorous outcrossing procedure must therefore be adopted before such mutants are subjected to further study in order to separate the mutation providing the desired phenotype from other coincidentally induced genetic lesions. Large mutant yields are however not

necessarily general with these mutagens. Although NTG is strongly mutagenic for bacteria (loc. cit.) and Neurospora crassa (Malling and de Serres 1967), and may induce 100% 'bleaching' of Euglena gracilis (McCalla 1965), Gillham (1965) was unable to demonstrate overlarge mutation frequencies of chromosomal or nonchromosomal genes of Chlamydomonas reinhardi with this agent.

Microbial geneticists are indeed fortunate that they are free to undertake experimental procedures in which they may discard the great majority of their cell populations without ethical, social, or economic qualms, a freedom obviously denied to workers with plant or animal materials. Conceptually, the only alternative to the profligacy presently practised by the microbial geneticist is an increase in the selectivity of mutation induction, or "mutagen specificity".

2. THE QUESTION OF SPECIFICITY.

a. Site Specificity.

The production of desirable mutations in chosen genes, leaving unaltered the remainder of the genetic complement, has been a goal for geneticists ever since mutations were shown to be inducible by physical or chemical agents. When genes were thought to be constructed of protein, the specific interaction of gene and mutagen seemed feasible, by analogy from immunological specificity. The subsequent implication of DNA as the genetic material (Avery, MacLeod, and McCarty 1944, Hotchkiss 1949, Hershey and Chase 1952) greatly diminished hopes of a mutational specificity based on the primary reaction

of gene and mutagen, particularly as genes were now shown to be composed of only four nucleotide bases with equivalent amounts of adenine and thymine, and of guanine and cytosine.

If mutations are produced solely by the interaction of mutagen and DNA, the overall similarity in composition of genes and not their unique base sequences should yield a random distribution of mutations. It has been clearly demonstrated however that mutants arising in the rII region of bacteriophage T4 are not at all randomly distributed, whether spontaneously occurring or induced by 5-bromouracil. Mutations occurred with greatest frequency at specific sites, "hot-spots", the location of these sites differing for the two classes of mutation - spontaneous or 5BU-induced (Benzer and Freese 1958). Despite this great site specificity, no significant differences were noted between spontaneous and induced mutant classes either in the proportion of rII mutants among all r type mutants isolated, or in the relative proportions of rIIA and rIIB mutants. There is therefore no evidence in these valuable studies of a cistron specificity of possible practical application. Some specificity in mutation induction may well be shown by such factors as the chemical preferences of mutagens for particular bases (reviews - Krieg 1963, Orgel 1965, Freese E. and Freese E.B. 1966, Zamenhof 1967, Singer and Fraenkel-Conrat 1969), the different types of mutation inducible (viz. transition, transversion, addition-deletion, and grosser chromosomal aberrations), and the relative phenotypic importance of mutation at different sites within a gene (e.g. Langridge 1968). These site specificities for mutation

may be explained at the level of the original mutagen-DNA interaction if the base sequence surrounding a "hot-spot" contributes in some way to its mutability, or if the unique configuration of DNA in a bacteriophage head leads to the preferential offering of sites for mutation. However, such site specificities have also been demonstrated in the Eucaryotes Schizosaccharomyces pombe (Gutz 1961) and Neurospora crassa (Ishikawa 1967). Contemporary workers are continuing the study of the influences of neighbouring bases on the mutability of sites (Koch and Drake 1968, Drake - unpublished results) and on their recombination (Drapeau, Brammar, and Yanofsky 1968, Berger, Warren,and Fry 1969).

b. Forward and Reverse Mutation.

Forward mutation involves the production of a new phenotype. It may represent either an additional cellular activity, as with mutation to drug resistance, or the diminishing or deletion of a cellular activity, as with mutation to auxotrophy from prototrophy. It is evident that, for the latter category at least, this change in phenotype may result from mutation at any of a large number of sites in one or more genes. It would therefore appear that mutagen specificity for forward mutation requires the specific recognition of genes by a mutagen. There is no evidence for this improbable event (Benzer and Freese 1958).

Reverse mutation on the other hand requires the restoration to wild type of a phenotype resulting from a previous forward mutation. In practice, there are three ways

by which this alteration in phenotype may be brought about. Firstly, the original forward mutation may be "corrected" back into wild type genotype by true reversion. Secondly, wild type phenotype may be restored by the substitution of another mutation at the site of the original forward mutation. This will leave the site still mutant with respect to wild type, but with partial or total wild phenotype (e.g. Guest and Yanofsky 1965). Thirdly, and paradoxically by a second forward mutation, intra- or extra-genic suppressor mutations may correct the effect of the first and still existent forward mutation with restoration of wild type phenotype, once again, in whole or in part (e.g. Berger, Brammar, and Yanofsky 1968, Garen 1968). These suppressor mutations may be detected by genetic means, either by the reisolation of the original forward mutation, or by the demonstration of suppressor activity for other mutations of a similar nature. For example, some phage mutations are (conditionally) lethal unless the phage is propagated in bacterial strains bearing a mutation suppressing nonsense (Benzer and Champe 1962, Garen and Siddiqi 1962, Epstein et al. 1963, Notani, Engelhardt, Konigsberg, and Zinder 1965) or missense (Reid and Berg 1968, Regos and Szende 1968).

Mutagen specificity for reverse mutations could involve the specific interaction of a base whose replacement was desired with a mutagenic agent chosen to bring about this replacement. There is strong evidence for this kind of reverse mutation site-specificity in bacteriophage T4 (e.g. Freese 1964) and in Salmonella typhimurium (Eisenstark et al.

1965). Tessman and his coworkers have shown an apparent specificity of hydroxylamine for cytosine when applied to phage S13 in vitro. In vivo hydroxylamine treatments of the phage however induced all 4 types of transition. Obviously the site specificity demonstrated by this mutagen depends upon the treatment conditions (Tessman, Ishiwa, and Kumar 1965). Different site specificities have also been demonstrated for the mutagens nitrous acid, 5BU, and NTG with the two phages S13 and T4 (Champe and Benzer 1962, Howard and Tessman 1964, Baker and Tessman 1968). As these specificities have thus been shown to depend on mutagen, conditions of treatment, and organism, it is obviously most important that generalisations should not be derived from such specialized circumstances. Lest it be thought that specificity be related to the complexity of organisms, the action of NTG in bacteriophage S13 has been shown more akin to that in Salmonella typhimurium than in another bacteriophage, T4 (Eisenstark et al. 1965, Baker and Tessman 1968). Complex organisms may however show a reduction of such specificity by formation of extragenic suppressor mutations, by modification of the mutagen in the intracellular environment, and by the availability of a greater range of other factors of potentially mutation-modifying nature.

Fortunately for our hopes of directed mutation, and perhaps in the teeth of hypothetical predictions, mutagen specificities for both forward and reverse mutation have been demonstrated in many organisms. Not only are these based on such factors as the differential activity of related mutagenic

agents, the types and relative proportions of genetic damages induced by them, or their specificities of action with regard to site, allele, locus, chromosomal region, cell, sex, strain, and organism (review - Auerbach 1966), but on interference with the result of the primary and essential mutagen-DNA interaction at some subsequent level. The processes of establishing a newly mutated allele in a cytoplasm coincidentally treated with mutagen have been likened by Auerbach (1965) to the passage of an object through a series of sieves, each possessed of the potential to prevent passages of the object. Whether or not this inhibition takes place may depend on the nature of the sieve, its cellular environment, and the effects of coincident mutagen treatment upon it. The object referred to is of course the establishment of the mutated allele in a clone of fully mutant cells.

3. THE ROLE OF ANCILLARY FACTORS IN THE INDUCTION OF MUTATION IN MICROORGANISMS.

What are the pathways to establishment of mutated genes, and at what steps may we lay down our sieves to interfere with them?

1. Genes may be shown to vary in sensitivity to mutagen dependent on their states of repression, derepression, or replication. Evidence has been presented by Kölsch and Starlinger (1965) that the ability of E. coli to synthesise β-galactosidase varies in its UV-sensitivity dependent on the functional state of the genes concerned. Such variable sensitivity of gene function to a known mutagen may well also apply to its mutability: a short abstract to this effect has

already been published (Lipschutz, Falk, and Avigad 1965), and it is believed that more extensive experimental data may soon be forthcoming from other workers. Evidence has also been presented that the sensitivity of DNA to the chemical mutagen NTG is greatest at the point of replication (Cerda-Olmedo, Hanawalt, and Guerola 1968), a factor which enhances the recovery of closely linked multiple mutants (Hirota et al. 1968).

2. Once a potentially mutagenic lesion has been formed, it must be able to participate in the new mutant activity. Ultraviolet studies with strains of E. coli suggest that UV-induced premutational lesions may not be considered immune from cellular dark repair and PR mechanisms until the first postirradiation round of DNA synthesis (Haas and Doudney 1959, 1960, Witkin 1963) and perhaps even later (Bridges and Munson 1968). It is evident that interference with these repair mechanisms may directly affect yields of induced mutation, and perhaps even spontaneous events. Kölsch and Starlinger (1965) have already shown that susceptibility to PR of an ultraviolet-inactivated gene depends upon its functional state of repression or derepression. Interference with repair mechanisms has been made at the genetic level by the use of mutants with increased or decreased repair ability (e.g. Witkin 1966, 1968), or at the phenotypic level by the addition of such repair inhibitors as caffeine (Witkin 1958, 1968) or acriflavine (Witkin 1961). These two repair antagonists appear to inhibit different steps of the repair processes, thus yielding a combined additive effect (Shankel and

Kleinberg 1968). Exploration of the nature, substrates, and mode of action of these repair processes constitute a research growth field of exhaustive and frequently repetitive documentation. Recent reviews of relevant aspects of photochemistry and photobiology have been written by, inter alia, Bridges and Munson 1968, Doudney 1968, Giese 1968, Hanawalt 1968, and Setlow R.B. 1968. Many workers have drawn attention to repair cross-sensitivities between some UV- or ionizing-radiation-induced lesions and those induced by some chemical agents, whose role in mutagenesis is thus similarly open to possible modification by nonspecific repair processes. In addition, the ability of cells to perform repair may vary at different stages of the life cycle, (e.g. Davies 1965, 1967), perhaps due to a variable quantity of repair enzyme (e.g. Boling and Setlow J.K. 1967).

3. The response to small changes in environment of the enzymes and structural machinery involved in replicating, transcribing and translating the genetic message is frequently a loss of fidelity. From an extensive literature, examples of such errors induced by potentially mutagenic agents include post-irradiation permeability changes with resulting loss of ions (e.g. Pollard and Weller 1968), inactivation or complete loss of nonchromosomal genetic material, destruction of intracellular pool compounds, interference with energy supply (e.g. Liapunova and Galtsova 1968), enzyme inactivation (e.g. Rowley and Newcombe 1964), release of a ribonuclease from ribosomes (Kucan 1966), or ribosomal inactivation (e.g. Lingens, Sussmuth, Wacker, and Chandra 1967), reduction of one or more

activities of tRNA (e.g. Aoki, Ikemura, Fukutome, and Kawade 1969), miscoding induced by nucleic acid photoproducts (e.g. Grossman, Ono, and Wilson, 1965), mRNA inactivation (e.g. Swenson and Setlow 1964), reduction of the ability of DNA to prime RNA or DNA polymerase activities (e.g. Ruddon and Johnson 1968), and an increase in the error frequency of irradiated RNA polymerase (Goddard, Weiss, and Wheeler 1969). Alterations in the fidelity of DNA polymerase are known to occur following genetic changes (e.g. Drake, Allen, Forsberg, Preparata, and Greening 1969), and it is not inconceivable that mutagen-treated DNA polymerase may itself become a mutagenic agent, even if of temporary duration. In addition, cross-linkage between cellular components by a known mutagen (UV) has been demonstrated (Smith, Hodgkins, and O'Leary 1966), and treatment of the medium used to suspend cells may itself interfere with synthetic processes (e.g. Pollard, Ebert, Miller, Kolacz, and Barone 1965).

Although most of these results have come from in vitro studies at dose levels far higher than any likely to be used in in vivo experiments, with the exception of peculiarities such as Micrococcus radiodurans, it is quite clear that interference of mutagens with cellular structures or mechanisms other than DNA may present several potential levels at which selection for or against newly mutant alleles may occur. Such treatments may also affect the modification of mutational damage by repair mechanisms which are, of course, similarly exposed to mutagen (Patrick and Haynes 1964, Kilbey 1969).

4. Immunity to repair and acquisition of function are in themselves not sufficient to establish a new mutation, which must subsequently participate in the formation of a completely mutant cell, and later of a clone of such cells. Stress against the new mutation may be exerted during segregation of mutant and nonmutant loci and in cell division. Delayed cell division, particularly if due to delayed DNA synthesis, may increase the time available for repair of premutational lesions. There may also be a simple necessity for the mutation to become established before the cell bearing it 'dies'.

5. Interaction may occur between the new mutant cell and components of the growth medium used for recovery of it and its progeny. Clarke (1962) provides a good example of such an interaction from his studies on Schizosaccharomyces pombe. Both ad^+ and met^+ revertants were induced by nitrous acid with similar frequency from an adenine and methionine diauxotroph, whereas UV induced met^+ but very few ad^+ revertants. Analysis revealed that methionine obligatorily added to the plating medium was selecting against ad^+ revertants produced by UV but to a much less extent against those induced by nitrous acid. Gratuitous methionine produced a similar reduction of UV-induced ad^+ reversion from the ad^- monoauxotroph, but in the absence of methionine, this strain yielded a large number of UV-induced ad^+ revertants. As reconstruction experiments showed that completed revertants grew well on methionine-supplemented agar, the inhibitory action of methionine on mutation induction to ad^+ must occur at some stage preceding the formation of a completely mutant cell.

Another such instance derives from the attempts of Zetterberg (1961, 1962) to induce auxotrophic mutants of Ophiostoma. Although histidine-requiring mutants could be recovered on complete medium after induction with NMU, none at all were recovered after UV-induction unless the complete medium was replaced by a histidine-supplemented minimal medium. With this alternative plating medium, both NMU- and UV-induced histidine auxotrophs could be recovered. Obviously components of the complete medium were preventing recovery of the desired mutants. A similar discrimination may exist in Neurospora crassa (Lein, Mitchell, and Houlihan 1948, Haas, Mitchell, Ames, and Mitchell 1952) although the general paucity of amino acid auxotrophs in Ophiostoma (Zetterberg 1961, 1962) is not paralleled in the second fungus (Barrat and Ogawa 1966). Zetterberg et al. (1969) has recently isolated a tryptophan auxotroph of Ophiostoma on minimal medium supplemented only with the desired amino acid. Growth of this UV-induced mutant was likewise inhibited on complete medium. A similar inhibition of guanine auxotrophs was earlier reported by Fries (1950).

Ions are effective in this regard as well as specific nutrients. The addition of manganous chloride to mutant recovery medium selectively inhibited growth of 8-azaguanine-resistant mutants of Penicillium notatum when induced with HN_2, but not when induced with X-rays, UV, or DES. HN_2-induced 7-azaindole-resistant mutants were not inhibited, however, thus demonstrating that this very interesting specificity was not solely dependent on the mutagen (Arditti and Sermonti 1962).

The presence of ammonium salts in the mutant recovery medium has been shown to prevent recovery of certain classes of arginine auxotrophs in Chlamydomonas reinhardi (Loppes 1969).

6. Further possibilities of modifying induced mutation frequency occur at the level of the background genotype. As this topic will be examined in the DISCUSSION, only brief mention of it will be made here. It is evident that the presence of mutations elsewhere in the genome is most unlikely to affect the primary reaction of studied allele and mutagen. Exceptions to this principle include mutations affecting access of mutagen to DNA, and the effects of neighbouring bases on site mutability (Drake - unpublished results). Such genetic background effects are apparent however, and almost certainly reflect an effective modification of a potential mutation. For example,in E. coli, a given tryptophan allele was mutable by DEB only when the strain was also auxotrophic for arginine (Glover 1956). Another tryptophan allele became UV-stable in a streptomycin-dependent (Witkin and Theil, 1960) or adenine-requiring background (Chopra 1967), this adenine requirement also preventing its spontaneous mutation. This last case is the only one for which nutritional influences at a phenotypic level have been denied responsibility. The spontaneous and induced reversions to try^+ are known to be of 2 suppressor classes and one nonsuppressor class (Bridges, Dennis, and Munson 1967), all of which are inhibited by the adenineless background. Such events can only be explained at a level subsequent to mutagen-DNA interaction.

As a final example in this section, isolation of auxotrophs of the tryptophan operon in Salmonella typhimurium

was greatly enhanced by the presence of a mutation (designated 47S) in the try A gene (Riyasaty and Dawson 1967). Over 50% of spontaneously occurring auxotrophs were anthranilic acid-requirers in contrast to about 5% in the 47S⁻ strain, and a great increase was also noted of indole and trytophan auxotrophs.

From these few examples, it is evident that modification of mutation frequency can arise from many events ancillary to the induction of a potentially mutagenic lesion in DNA. A compendium of these and other examples is given in Table 1.

4. A SPECIFICITY OF ANCILLARY EFFECTS ?

Ancillary effects may be superimposed upon a differential response of two or more genes to a particular mutagen, or the different responses of a single gene to a number of mutagens, singly or in combination. It is evident that these effects should, at least in theory, exert their action in one or more ways:

A By an effect to the same extent upon both the lethality and induction of mutation produced by a mutagenic treatment. This should appear as a dose-modifying response to the ancillary condition, as might be expected if the lethal and mutagenic damages induced by the agent in question were the same, or, if different, similarly subject to modification. For repair mechanisms at least, survival changes probably represent the overall efficiency of repair of potentially lethal damages in all genes. Alteration of mutation frequency on the other hand will depend on the efficiency of repair of premutational damage in different loci (Witkin 1966),

TABLE 1. A COMPENDIUM OF AGENTS MODIFYING SPONTANEOUS OR INDUCED MUTATION FREQUENCY IN MICROORGANISMS.

TIME OF ACTION OF AGENT	MUTAGEN USED.	DIRECTION OF MUTATION	NATURE OF MODIFYING AGENT	REFERENCES CITED
	UV	R	Preparative Growth	Witkin 1956
	UV	R	Medium	Tabaczynski 1962
BEFORE	X rays	R	" "	Munson & Bridges 1964
MUTAGEN	UV	R	Photoprotection	see Genetic Background (Repair)
TREATMENT	EMS, propane-sultone	R	$MnCl_2$, $CoCl_2$	Bohme 1962
CONTEMPORARY	UV	R	Temperature	Kilbey 1963
WITH	UV	R	"	Ashwood-Smith & Bridges 1967
MUTAGEN	X rays	R	"	Munson & Bridges 1964
TREATMENT	HNO_2 and alkyl nitrosamides	R	"	Zimmermann et al. 1965.
	UV	R	Composition of suspending liquid for conidia-whether pH or phosphates responsible unknown.	Allison (unpublished)

TABLE 1. continued

TIME OF ACTION OF AGENT	MUTAGEN USED	DIRECTION OF MUTATION	NATURE OF MODIFYING AGENT	REFERENCES CITED
	UV	R	Composition of suspending liquid for conidia - due to chloride ions	Matsuyama, Namaki, Okawawa & Kaneko 1964
CONTEMPORARY WITH MUTAGEN TREATMENT (Contd.)	UV, X rays	R	Dose fractionation	Kada, Doudney & Haas 1966
	DEB	R	" "	Auerbach (in press)
	NTG	F	pH	Adelberg, Mandel, & Chein Ching Chen 1965
	HNO_2, alkyl nitrosamides	R	"	Zimmermann et al. 1965
	DEB, UV	R	Combined Mutagen-Interaction Treatment	Auerbach & Kølmark 1960
	UV, X rays	R	" "	Kada, Doudney, & Haas 1966
	UV, gamma rays	R	" "	Davies, Arlett, Bridges & Munson 1967
	UV	F	Dose-Rate-Dependence of Kinetics of Mutation Induction	Novick & Szilard 1951
	DEB	R	" " "	Kølmark & Kilbey 1962

TABLE 1. continued

TIME OF ACTION OF AGENT	MUTAGEN USED	DIRECTION OF MUTATION	NATURE OF MODIFYING AGENT	REFERENCES CITED
	Many	R,F	Residual Division and Medial Supplementation	reviews - Kimball 1966, Witkin 1966, 1967.
	UV	R	Incubation Temperature	Witkin 1956
	X rays	R	" "	Munson & Bridges 1966
	HNO_2	R	" "	Zimmermann et al. 1966
AFTER	HNO_2	R	" "	Auerbach & Ramsay 1968
MUTAGEN	EMS	R	Pulse heating circa 50°C.	Strauss 1962
TREATMENT	UV	R	" " " "	Sideropoulos, Johnson, & Shankel 1968
	DEB	R	After-effect of mutagen	Kølmark & Kilbey 1962
	EO	R	" " " "	Kilbey & Kølmark 1968
	UV, NMU	R F	Presence of "normal" nutrients	Clarke 1962 Zetterberg 1961, 1962
	$MnCl_2$	R	Presence of other salts	Demerec & Hanson 1951
	UV, X rays, chemicals.	R	Presence of Phenotypic Inhibitors of Repair	see Genetic Background (Repair)
GENETIC BACKGROUND EFFECTS	Many	R, F	Dark Repair Processes	reviews - see Jagger 1964 Kimball 1966, 1968 Witkin 1966, 1967 Hanawalt 1968, etc.

TABLE 1. continued

TIME OF ACTION OF AGENT	MUTAGEN USED	DIRECTION OF MUTATION	NATURE OF MODIFYING AGENT	REFERENCES CITED
	UV	R,F	Photorepair	Setlow (J.K.) 1966. Hanawalt 1968 etc.
GENETIC BACKGROUND EFFECTS - continued	spont. UV, EMS, DEB.	R,F	Miscellaneous Effects of Genetic Background	Glover 1956, Witkin & Theil 1960, Böhme 1963, Clarke & Loprieno 1965, Mohn & Kaplan 1967, Chopra 1967, Chopra & Purnima 1968, Riyasaty & Dawson 1967.
	DEB, EMS	R	Alteration of Kinetics of Mutation Induction	Allison 1968
	UV	R		Chang, Lennox, and Tuveson 1968
MISCELLANEOUS EFFECTS	UV	R	Exhaustion of Medial Energy Source	Grigg 1958
	DEB	R	Developmental Stage of Culture (or dose rate)	Auerbach & Ramsay 1968

Abbreviations used:

Direction of Mutation - Reverse (R) or Forward (F).

although over the whole genome this might correspond to that of survival. The findings by Kilbey and de Serres (1967) and Kilbey (1967) that reversion of many different mutants of three different loci in Neurospora crassa could be photoreactivated to the same extent as survival confirms this viewpoint, although it does not rule out the existence of specificities for certain other loci. PR acts only upon a single class of substrates, the pyrimidine dimers, which may be responsible to roughly the same degree for UV-induced mutation of the loci concerned and lethality.

B. By an effect greater upon mutation induction than on lethality (or vice versa). Examples of this type include the most interesting finding by Kilbey (1967) of just such a specificity as predicted in A. An allele of inositolless was photorepaired to a much lesser degree than survival. Explanations for this phenomenon have been discussed by the discoverer, including a possible difference of lesion as well as of repairability. Other examples of this type include the posttreatment modification effects producing MFD in UV-irradiated E. coli strains, and the demonstration that whereas UV killing in these strains is reactivable by direct PR, mutation of certain auxotrophies to prototrophy and of lac^+ to lac^- is not (Witkin 1966). In addition, Kondo and Kato (1968) have demonstrated that uvr^- mutants of a strain of E. coli phr^- are about 25 times more sensitive to killing by UV or 4NQO than their uvr^+ parents, but about 35 times as sensitive to the induction of arginine reversions by these agents. In Chlamydomonas reinhardi, mutation to UV sensitiv-

ity increases mutation frequencies at low doses of UV although the maximal mutation frequency recovered from the mutant strains never approaches that of wild type. PR restores equivalence of induced mutation frequencies of wild type and mutants (Davies and Levin 1968), and a differential activity of repair mechanisms on mutagenic and lethal lesions is implied. Further studies on phenomena such as these could lead to the provision of more examples for section C.

C. By an effect greater upon certain mutations than upon others. PR of the adenine and inositol alleles already described (Kilbey 1967) falls in this section if a comparison is made between the alleles, but these are many other examples of this type of modification. Bridges, Dennis, and Munson (1967) have demonstrated, following up an original suggestion of Witkin (1963), that the reversions to try^+ of E. coli B/r WP2 try^- which demonstrate a broth effect and MFD are suppressor mutations whilst the nonsuppressor mutations demonstrate no such responses. Witkin (1966) has demonstrated that mutation of this same strain WP2 to its ultraviolet - sensitive, excisionless derivative $WP2_s$ causes at the same survivals twice the frequency of UV-induced try^+ in the parent on broth-supplemented medium, although this ratio can be drastically altered by using a plating medium which allows MFD to take place in the parent, the mutant being incapable of MFD under normal conditions. The parent strain however demonstrates at equivalent survivals only one-tenth as many mutations to streptomycin-resistance as the mutant. This suggests that the mutation to uvr^- has revealed differences in the repair-

ability of some mutations relative to others. As Witkin has also demonstrated that about 90% of the UV-induction of try$^+$ revertants or streptomycin-resistants in WP2 are photoreactivable, combination for comparative purposes of these mutation systems with the definitely nonphotoreactivable lac$^+$ to lac$^-$ system reported in B. should in theory at least reveal considerable mutagen specificity.

In a further example, Chang, Lennox, and Tuveson (1968) have shown very different ratios of reversion of different loci in an Aspergillus nidulans diauxotroph dependent on the UV-sensitivity of the strains bearing the markers.

An effect of genetic background has been demonstrated by Chopra (1967) with the same strain WP2, where the try$^-$ allele became stable to spontaneous or induced reversion after the addition of a second requirement, for adenine; whereas the try$^-$ marker acquired stability, the ad$^-$ marker reverted normally. An inhibitory effect of methionine on UV-induced adenine reversion in Schizosaccharomyces pombe (Clarke 1962) may be due to the fact that UV-induced reversions are predominantly suppressor mutations at distant loci. Nitrous acid-induced reversions, which showed much less methionine inhibition, are predominantly either closely linked suppressor mutations or true reversions. It is therefore conceivable that methionine acts in a grossly inhibitory way only upon UV-induced mutations in the distantly sited suppressor genes. This explanation was put in doubt by the finding (Clarke 1969) that NMU-induced ad$^+$ revertants, which are also strongly methionine-inhibited, are not due to such distantly situated

suppressor mutations. An effect of the inducing mutagen upon the expression of reversions may provide a valid alternative explanation.

Finally in this section, but most studied, is the adenine and inositol reversion system in Neurospora crassa, discovered originally by Kølmark (1953), and exploited since then by him, Auerbach, and members of her group. As well as characteristic response curves to a number of mutagens, a variety of ancillary conditions have been found to alter the ratio of inositol to adenine reversions given by treatment with a particular mutagen. These conditions include PR, dose dependence, stage of growth, genetic background, temperature of treatment and of posttreatment incubation, composition of liquid used for suspension of conidia, and combination treatments with other mutagens (Allison 1968, 1969, Auerbach and Kølmark 1960, Auerbach and Ramsay 1967, 1968, Kilbey 1963, 1967, Kølmark and Kilbey 1962, Malling, Miltenburger, Westergaard, and Zimmer 1959).

Of greatest interest to mutation workers are sections B and C, whose very existence is sufficient justification for further investigation.

5. EFFECTS OF MEDIAL SUPPLEMENTATION, RESIDUAL SYNTHESIS, AND DIVISION.

Since the earliest report by Demerec (1946) of an increased yield of bacteriophage-resistant mutants following postirradiation preselection divisions, similar reports have been made for the induction by UV of auxotrophic (Davis 1948),

prototrophic (Davis 1950, Demerec and Cahn 1953), and streptomycin-independent mutants (Labrum 1953) of E. coli. In the Introduction to a paper published in 1956, Witkin wrote "this "delayed appearance" of induced bacterial mutants has become one of the hardy perennials among the unsolved problems of microbial genetics". The now classical report which followed this opinion was of the first systematic attempt to shed light on the problem.

A delayed appearance was inferred in many instances from the increased mutation frequency noted on incorporation into the selective medium of small quantities of growth-promoting nutrients, either singly or in combination. Witkin (1956) cited five possible explanations to account for this delayed appearance of induced bacterial mutants, namely delayed mutation, phenotypic lag, and segregation of nuclei or chromosomal strands (proposed by Demerec 1946), irregularity in growth of newly mutant cells (proposed by Newcombe and Scott 1949), and the delayed division of newly induced mutants (proposed by Ryan 1954). A sixth proposal referred to a possible methodological artefact which might prove responsible for the delay of bacteriophage-resistant mutants already observed (Newcombe 1953).

In her report, Witkin concluded that for the UV mutagenesis of strains of Salmonella typhimurium and E. coli none of these postulated explanations held true. She showed that mutational yields of reversions to prototrophy of a number of auxotrophic strains were low if protein synthesis was inhibited by an insufficient quantity of amino acids, or

in the presence of the antibiotic chloramphenicol. The effect of amino acid deprivation was particularly marked with cells prepared in nutrient broth, but very much less with cells grown in a minimal medium. That these effects acted upon the mutation processes was indicated by the absence of appreciable survival changes during the period of altering mutation yield. As she herself had earlier shown (Witkin 1953), increase or decrease of postirradiation incubation temperature yielded fewer mutants if the changed temperature was maintained throughout, but rapid switches in temperature could return higher mutation yields. In a series of experiments in which the time of application of posttreatments was varied, it was demonstrated that the effects on protein synthesis were apparent only during the first post irradiation hour, that is, probably until the first postirradiation doubling of DNA in her system. Witkin (1956) offered in explanation either a repair of mutagenic lesions, such repair being a prerequisite for survival of a mutant, the instability of a mutagenic intermediate whose quantity or stability depended upon protein synthesis, or an instability of the mutagenic lesion in the gene. A premutational nature for ultraviolet-induced lesions was suggested by the effects of such posttreatments as visible light, temperature, metabolites, and antimetabolites.

Contemporary with these studies by Witkin (and others), and in continuation of previous investigations on the induction of bacterial mutation by UV-irradiated organic substrates (Haas, Clark, Wyss, and Stone 1950), Haas and Doudney (1957)

found that supplementation of the preirradiation growth medium of E. coli strains with, inter alia, the purines and pyrimidines found in RNA greatly increased their recovered yields of fermentation mutants. They suggested that an intracellular alteration of these nucleic acid precursors by UV might lead to mutation induction when the precursors were eventually incorporated into nucleic acid. A relationship between conditions affecting protein synthesis and eventual mutation yield was also shown by these workers. They then embarked on a study of various posttreatment effects on mutation yield to prototrophy (Doudney and Haas 1958, 1959, 1960, Haas and Doudney 1958, 1960). In summary, their findings were that bacteria incubated after UV-irradiation in a minimal medium lacking a nitrogen source with subsequent plating on a selective agar supplemented with 2·5% nutrient broth (the 'high-yield' plating medium) gave a decreasing mutation yield as the posttreatment was prolonged. Conversely, posttreatment incubation in amino acid-rich medium gave an increase in mutation frequency. Further addition of chloramphenicol to the amino acid-rich medium gave a decline in mutation frequency comparable to that observed in nitrogen-deficient minimal medium. With a minimal plating medium however, a high yield of mutants was only recovered when a posttreatment incubation in amino acid-rich medium was given until, or just after, DNA synthesis, and before cell division.

From these experiments, a terminology was derived by Doudney and Haas (1958, 1959, 1960). "Mutation stabilisation" was determined by incubation of UV-irradiated cells in an

amino acid-rich medium with subsequent plating on broth-supplemented agar. The amino acids exerted their effect of maintaining or increasing mutant yields before RNA or DNA synthesis was evident. If this mutation stabilisation did not occur, as when the cells were incubated in the same amino acid-rich medium with added chloramphenicol, "potential mutations" were removed by a process of "mutation frequency decline" (MFD). Doudney and Haas (1960) showed that whatever the processes involved in MFD, it could be completed in the absence of protein, RNA, or DNA synthesis, and, further, that its occurrence did not materially influence the rate of subsequent synthesis of these macromolecules. Recently Kimball (1966) has pointed out that the kinetics of MFD should not necessarily be interpreted as implying the completion of the process during the period of applied posttreatment, as this completion may just as well occur on the plating medium after the initial step has occurred during posttreatment.

That the final establishment of a UV-induced genetic change occurred at the time of postirradiation DNA synthesis was suggested by three observations. Firstly, induction of prototrophs from jointly amino acid- and thymine-requiring strains of E. coli showed that UV-irradiated cells remained prone to MFD in the presence of amino acids but in the absence of thymine. Addition of thymine then led to increasing resistance to MFD, as did the presence of amino acids and thymine from the outset (Weatherwax and Landman 1960, Witkin 1963, Doudney 1963). Secondly, the recovery of a high yield of induced prototrophs on a minimal plating medium after

incubation in amino acid-rich medium occurred only if this intermediate incubation was prolonged until, or just after, DNA synthesis. This was termed "mutation expression" by Haas and Doudney (1958). "Mutation fixation" was signalled by the immunity of mutation frequency to a chloramphenical posttreatment previously able to alter this frequency. Thirdly, "mutation incorporation", or the loss of PR of mutation after incubation in amino acid-rich medium occurred at about the same time that DNA synthesis occurred (Doudney and Haas 1960, Haas and Doudney 1960), and also at about the same time that the cells became immune to the MFD-promoting effects of amino acid deficiency (Witkin 1963).

These complex studies have been reviewed by one of the participants (Doudney 1968) and in a more critical manner (albeit sympathetically) by Kimball (1966). In 1962, Doudney abandoned the hypothesis that UV-induced mutations were due to the incorporation of radiation-altered RNA precursors into RNA (Doudney and Young 1962). This RNA (with protein attachments) was supposed to evoke copying errors when used in DNA synthesis as an intermediate template, perhaps only after UV-irradiation (Doudney and Haas 1958, 1960, Doudney 1961). The primary reason for this jettisoning of the hypothesis was the accumulating evidence of Witkin (1958, 1961, 1963) that the UV-induced premutational lesion was situated in the DNA. This evidence came from postirradiation studies, including some with basic dyes with great affinity for DNA; these dyes served also to inhibit MFD under conditions of amino acid deprivation, and to enhance the mutagenic effect

of low doses of UV. The process of MFD was now tentatively interpreted as the workings of repair processes active on DNA but inhibited by these same basic dyes and by conditions which favoured RNA and protein synthesis. (Witkin 1961).

Ultraviolet photochemical studies soon provided strong evidence that thymine dimers were among these mutagenic lesions, perhaps to the exclusion of all others, although in more recent years responsibility has been stretched to include others kinds of pyrimidine dimer. In 1964, Setlow (R.B.) illustrated that the elimination of thymine dimers from DNA by secondary short wave ultraviolet radiation or enzymatic PR corresponded well with the recovery of biological activity of DNA and an increase in the survival of irradiated bacterial suspensions, determined as colony-forming ability. Action spectra had previously suggested that thymine dimers were responsible for UV-induced mutation in a bacteriophage (Setlow J.K. 1963). Since that time, the primacy of pyrimidine dimers as UV-induced mutational lesions has been questioned, although belief in their importance for killing remains unshaken. An UV-photoproduct other than a pyrimidine dimer has been demonstrated, at least in vitro, to be capable of "mutagenic activity", namely cytosine hydrate (Ono, Wilson, and Grossman 1965, Grossman 1968). The lack of PR apparent in such mutational systems as lac^+ to lac^- in E. coli (Witkin 1966) may be due to mutagenesis by such nonphotorepairable UV-products, although other explanations based on an inaccessibility to repair may be equally valid. The influences of direct PR and of the many components implicated in dark repair

have been reviewed by Setlow (J.K.)(1966) and Witkin (1966, 1968), with respect to their ability to repair potentially mutagenic lesions. Recent results have demonstrated an involvement of both excision-repair mechanisms (Witkin 1968) and bacterial recombination mechanisms in mutation induction by UV irradiation (Miura and Tomizawa 1968, Witkin 1969).

There seems little doubt that whereas enzymatic photo-repair mechanisms utilise as substrates only dimerized pyrimidines, the dark repair mechanisms may demonstrate a great catholicism of function, being capable of excising lesions induced by a variety of deleterious agents. This has been demonstrated both indirectly by cross-sensitivity of many mutants to radiation and chemical agents, e.g. Searashi and Strauss 1965, Reiter and Strauss 1965, Bridges and Munson 1966, Loveless 1966 (partial review), Malke 1968, Zimmermann 1968, Böhme 1968, Clarke 1969, Kilbey and Smith 1969, and by the direct demonstration of removal of mutagenic products from DNA, the first step of presumed repair at the molecular level, after treatment of cells with such agents as HN_2 (Hanawalt and Haynes 1965, Kohn, Steigbigel, and Spears 1965, Lawley and Brookes 1965, Loveless, Cook, and Wheatley 1965), sulphur mustard (Papirmeister and Davidson 1964), X-rays (Emmerson and Howard-Flanders 1965, McGrath, Williams, and Swartzendruber 1966), monofunctional alkyl sulphonates (Strauss 1963, Strauss and Wahl 1964), and NTG (Cerda-Olmedo and Hanawalt 1967). The subject has been partially reviewed by Haynes (1966).

It is important to emphasise that the demonstration of

an increased mutation frequency on a plating medium such as the broth-supplemented agar previously mentioned compared with that recovered on a minimal plating medium does not necessarily constitute evidence of the presence or action of modifying phenomena. There is ample evidence that some mutations actually require the division of cells for their establishment, despite the operations of the repair processes and the various nutritional means so far discovered by which they may be encouraged or inhibited. It has been shown by Margolin and Mukai (1961, 1964) with Salmonella typhimurium that 2AP-induced reversions of various alleles of leucine-less could be separated into two classes on the basis of a requirement, or lack of such requirement, for residual division before mutants could be isolated on a minimal plating medium. Among reasons put forward for this phenomenon were the induction of different classes of transition by 2AP, with different requirement for division, and a possible single-strandedness of the genetic message used during mutation expression for mRNA transcription. Further studies of this kind were reported by Ronen (1964) for DES-induced reversion of auxotrophs of Salmonella typhimurium, although delayed mutation may be responsible in this last instance. For bacteriophage T4, Krieg (1963) has demonstrated that some mutants yield a low frequency of EMS-induced reversion if selected directly after treatment. A cycle of nonselective growth before selection however showed these mutants to be in need of expression as they then yielded high reversion frequencies. Kirschmann and Davis (1969) have recently shown that

low-level-antibiotic suppression of conditionally-streptomycin or chloramphenicol-dependent auxotrophs required addition to the medium of a minute quantity of the previous growth requirement, sufficient to allow one residual division. This need for division before suppression could be manifested was undertaken by leaky auxotrophs without addition of growth requirement. Such residual divisions may also be necessary for suppressor activity when induced by mutation, at least in some instances.

From an operational viewpoint, therefore, it should be possible to separate mutations requiring residual division from those demonstrating an enhancement of mutation yield on plating media supplemented with a pool of amino acids (or broth). A stimulatory effect of small quantities of amino acid-rich supplements to selective media has been noted by many workers with a variety of mutagens and organisms, but in only a very few cases has the very important distinction been made between a requirement for residual synthesis and division, which may be satisfied by the addition of a small quantity of the previous growth requirement, or by its presence in a pool of amino acids or broth, and a genuine additional stimulation over and above this by a pool of amino acids. For the sake of clarity of discussion, these two effects will be referred to as the "broth-effect" and the "specific requirement-effect". A compendium of published results on this topic is given in Table 2. In no case in the literature has a true broth-effect been demonstrated for a mutagen other than ultraviolet light and for organisms other than Salmonella typhimurium and

TABLE 2. COMPENDIUM OF PUBLISHED RESULTS OF EFFECTS OF ADDITION OF BROTH OR AMINO ACID(S) TO MUTANT RECOVERY MEDIA, NOTING THE GROWTH CONDITIONS USED TO PREPARE CELLS.

ORGANISM USED	GROWTH PHASE	MUTAGEN USED	PRETREATMENT GROWTH MEDIUM	BROTH EFFECT PROVEN	BROTH EFFECT POSSIBLE	SPECIFIC REQUIREMENT EFFECT	REFERENCES CITED
S. typhimurium LT2.tryC-3$^-$	S	UV	broth	+	+	-	Witkin 1956
	S	UV	minimal	+	+	+	Witkin 1956
E. coli K12, try$^-$pur$^-$	ON	UV	broth	-	-	+	Tabaczynski 1962
	ON	UV	minimal	?	?	+	
E. coli B/r WP2 try$^-$hcr$^+$	L	UV	minimal	+	+	?	Munson & Bridges 1966
try$^-$hcr$^-$	L	UV	minimal	+	+	?	
E. coli B/r WP2. try$^-$hcr$^+$	L	X rays	minimal	-	-	?	Bridges (pers. comm.)
E. coli. B/r WWP2 try$^-$hcr$^+$	S	UV	broth	+	+	+	Clarke 1967
try$^-$hcr$^-$	S	UV	broth	+	+	+	Clarke 1967
E. coli B/r WP2. try$^-$hcr$^+$	L	gamma	minimal	-	-	?	Munson & Bridges 1966
try$^-$hcr$^-$	L	gamma	minimal	-	-	?	Bridges, Law, & Munson 1968
E. coli B/r WP2. RRU4 try$^-$ thy$^-$hcr$^+$	L	thy$^-$	minimal	?	?	-	Bridges, Law, & Munson 1968

TABLE 2. continued

ORGANISM USED	GROWTH PHASE	MUTAGEN USED	PRETREATMENT GROWTH MEDIUM	BROTH EFFECT PROVEN	BROTH EFFECT POSSIBLE	SPECIFIC REQUIREMENT EFFECT	REFERENCES CITED
E. coli B/r try$^-$ (not WP2)	S	UV	minimal	?	+	?	Strauss & Okubo 1960
	S	DES	minimal	?	+	?	
	S	epichloro-hydrin	minimal	?	+	?	
E. coli K12. T71. arg$^-$	S	Hydroxyl-amine	minimal	?	+	?	Lie 1964
E. coli K12. T71. threo$^-$	S	UV	minimal	?	+	?	Lie 1965
E. coli WWU. arg$^-$ (multiple auxotroph)	L	tritium decay	minimal	?	+	?	Person & Bockrath 1964

Abbreviations:

GROWTH PHASE: L = logarithmic, S = stationary (24 hrs), ON = overnight (18 hrs.)

MUTAGENS: other than previously stated - thy$^-$ = thymine starvation

PRETREATMENT GROWTH MEDIUM: Broth = nutrient broth, minimal = minimal salts + required supplements only.

NATURE OF EFFECT: + = definite positive, - = definite negative, ? = untested or uncertain.

Broth Effect (Proven) = where a genuine broth effect has been established

Broth Effect (Possible) = where a stimulatory effect of broth has been noted, but that the effects were due to broth rather than to specific growth requirement in the broth not tested.

Broth Effect and Specific Requirement Effect - as defined in the text.

E. coli. The definite absence of a broth-effect has been noted in X- and gamma-ray-mutagenesis of E. coli. It has not been possible to decide whether a genuine broth-effect exists for mutagens other than UV and in other species of microorganisms.

An enormous concentration of research effort on radiation-induced mutation will be apparent from this review. Our knowledge of the mutagenic action of chemical agents, with especial reference to the modification of this action by ancillary factors, is sadly deficient.

6. THE GENERAL PROBLEM AND THE CHOICE OF SYSTEM.

It will be evident from the foregoing discussion that a lengthy catalogue can be compiled of the different ancillary effects acting on mutation induction. As the range of systems under investigation increases, it is highly probable that this list will be augmented. The unpalatable fact remains that in very few instances has a direct explanation been proferred for any of these observations, although hypotheses are abundant. Only with effects of repair mechanisms has further explanation been attempted both at deeper physiological and molecular levels, and with conspicuous success. Unless this collection of ancillary effects is to remain only a catalogue of interesting biological phenomena, analysis of their causes and mechanisms is essential. Although it is too early to hope that any one of them may prove worthy of general application, the matter cannot be resolved without further experiment.

For example, even the surface analysis of two apparently related observations may reveal totally different mechanisms. The description of a restriction of UV-induced mutation to try^+ in E. coli WP2 in the presence of a streptomycin-dependence marker (Witkin and Theil 1960) has been tentatively ascribed by Witkin (1965) to the interference of the drug with the efficiency of the suppressors responsible for the great majority of induced revertants in this strain (Osborn and Person 1967). A contradictory finding was reported by Glover (1956), but it appears that in this instance a different pair of streptomycin-dependent and tryptophanless alleles was used. Witkin's hypothesis may well prove to be true as a). streptomycin-dependence has in some cases been shown to be allelic with streptomycin-resistance (Luzzato, Schlessinger, and Apirion 1968), and b). streptomycin-resistance has been proved to interfere with suppressor efficiency in E. coli (Gartner and Orias 1966, Kuwano, Ishizawa, and Endo 1968, Otsuji and Aono 1968, Kuwano, Endo, and Ohnishi 1969). As only about 25% of spontaneously arising try^+ revertants were suppressors (Osborn and Person 1967), a correspondingly small reduction of the spontaneous mutability apparent in a streptomycin-dependent background might well escape notice without a special search, the number of mutants recovered being in any case low.

In contrast, interference with the efficiency of induced suppressors cannot be responsible (other than in part) for the reversion stability of the same try^- allele in an adenineless background (Chopra 1967). Here, where medial influences have been excluded from responsibility, the adenine

requirement totally prevented occurrence of all try^{+} revertants, whether suppressor or nonsuppressor, spontaneous or induced. Evidently the adenine requirement cannot be totally mimicking the action of streptomycin as it inhibits spontaneous reversion, although one can not exclude such a mechanism of action upon the suppressor revertants alone. It is much more likely that an explanation will be found at the level of expression of the relevant tryptophan gene. With this particular example, the absence of any apparent relationship between the synthetic pathways of adenine and tryptophan renders even hypothesis difficult.

These modifying processes must exert their influence under very complex circumstances, i.e. before, during, or after the induction of a potentially mutagenic lesion in the DNA, and in a cell cytoplasmically treated with mutagen as well as genetically, which must then give rise to viable progeny. Presumably the elucidation of these phenomena would be facilitated by the analytical approach of separating the processes of modification into their component parts. This requires that the investigator be able to treat genetic material with mutagen with or without cytoplasmic influence, and to assay its biological activity in mutagen-treated or untreated cytoplasm which may itself be subjected to other variations in genotypic and phenotypic background.

Such assay of biological activity is permitted by the property of some bacterial species to transfer genetic information by means of deoxyribonucleate preparations. This property may be accomplished by a limited number of

bacterial species, including B. subtilis, Diplococcus pneumoniae, Haemophilus influenzae, Moraxella sp., Neisseria sp., Rhizobium sp., Streptococcus sp., and Xanthomonas sp., and it has also recently been demonstrated in the Micrococci (M. lysodeikticus and M. radiodurans). Claims have also been made for its existence in fungi, for example, Allomyces sp., Aspergillus nidulans, Neurospora crassa, and Saccharomyces cerevisiae, although the evidence cannot be said to be compelling. Some tissue cells in culture are said to demonstrate the phenomenon of genetic change after exposure to deoxyribonucleate preparations from suitably marked donor cells, (e.g. Szybalska and Szybalski 1962, Podgajetskaya, Bresler, Surikov, Ignatova, and Olenov 1964, Glick and Salim 1967, Fox, Fox, and Ayad 1969). It is however unfortunate that the micro-organism best known for its physiology and genetics, E. coli, is capable only of a specialised kind of transformation involving simultaneous infection with a bacterial virus.

Although relatively high frequencies of transformation may be obtained from Diplococcus pneumoniae and Haemophilus influenzae, these organisms are nutritionally fastidious and allow only the use of drug and antibiotic resistances and the odd sugar fermentation as genetic markers. By comparison, B. subtilis strains will grow on simple chemically defined media, thus allowing the use of a wide variety of auxotrophic markers in addition to drug and antibiotic resistances. This property has already been exploited for the study of the transformation phenomenon per se, to provide an elementary genetic map of the organism, and for biochemical, recombin-

ational, and radiobiological purposes. Although in B. subtilis transformation frequencies are much lower than those obtained in Diplococcus pneumoniae or Haemophilus influenzae, the recent development of population fractionation systems for the selective enrichment of competent cells (Singh and Pitale 1967, Cahn and Fox 1968, Hadden and Nester 1968, Richardson and Leach 1969), the discovery of a soluble competence factor (Charpak and Dedonder 1965, Felkner and Wyss 1964, 1968, Akrigg, Ayad, and Barker 1967) and metabolic studies on the nature of competence (e.g. Bott and Wilson 1968) promises improvement in the near future.

Further improvement of transformation frequencies will come from the ability to ensure that cells competent for transformation are supplied with DNA donor preparations enriched for particular genetic markers (e.g. Saito and Masamune 1964, Ayad and Blamire 1968, Ayad, Barker, and Weigold 1968) or with "transfecting" preparations of bacteriophage DNA (review - Spizizen, Reilly, and Evans 1966, and a number of papers in Bacteriological Reviews 1968. vol. 32. no. 4. suppl. 1). Use of such bacteriophage DNA preparations would allow the investigator to exclude effects produced by mutagen or other treatments on the ability of the DNA to be recombined into the genomes of recipient cells, particularly when the phage genomes themselves may be isolated as single DNA molecules from certain phages (Okubo and Romig 1965, Reilly and Spizizen 1965, Riva, Polsinelli, and Falaschi 1968).

Before such transformation experiments are possible,

however, suitable mutants and mutation systems should be available. The derivation of such systems constitutes one aspect of the work presented here. Although there are numerous reports in the literature of the isolation of various kinds of auxotrophic, degradative, sporulation, suppressor, radiation-sensitive, and recombination-deficient mutants of B. subtilis, also of reputed base composition mutants, the workers concerned have in general been interested solely in the isolation of mutants, not in the efficiency with which they may be induced or recovered. Other publications record the isolation of bacteriophage mutants for B. subtilis host systems. The apparent mutagenicity of the transformation phenomenon itself has been documented, as have a number of studies on the inactivation of preparations of transforming DNA by mutagenic agents, and in some cases, the repair of this inactivation. Quantitative mutation experiments are however uncommon, those in the literature falling into three classes:

A. The experiments of Zamenhof and coworkers on the mutagenic effects of dry heat on spores (Zamenhof 1960, 1961, Chiasson and Zamenhof 1966), mutability of stored spores (Zamenhof, Eichhorn, and Rosenbaum-Oliver 1968), the comparative UV-induction of forward and reverse mutations in spores and vegetative cells (Zamenhof and Reddy 1967), the genetic mechanisms of radiation resistance (Zamenhof, Bursztyn, Reddy, and Zamenhof 1965), and the genetic control of mutation rates (Zamenhof, de Giovanni-Donnelly, and Heldenmuth 1962), (partial review - Zamenhof 1967).

B. Freese and coworkers have studied peroxides, hydroxylamine, and related compounds for their mutagenic and inactivating potential in vitro, with attempted analysis of the relevant chemical lesions (Freese E., Bautz, and Freese E.B. 1961, Freese E. and Strack 1962, Strack, Freese E.B. and Freese E. 1964, Freese E.B. and Freese E. 1964, Freese E.B. 1965, Freese E.B., Gerson, Taber, Rhaese, and Freese E. 1967). Bresler and coworkers have devoted themselves to a quantitative and comparative study of mutation and inactivation of DNA by many mutagens, singly and in combination (Bresler and Perumov 1962, Bresler, Kalinin, and Perumov 1964, 1967, 1968).

C. Four miscellaneous papers on in vivo mutagenesis by

i. Jensen and Haas (1963), who tried to derive support for the hypothesis held at that time by Doudney (loc. cit.) that photoproducts formed outside DNA were responsible for UV-induced mutation when subsequently incorporated into nucleic acid templates. Jensen and Haas showed that DNA from an auxotroph irradiated in vivo yielded no mutation-transformants when extracted immediately after irradiation and used to transform the same auxotroph. Mutation-transformants were obtained when the irradiated donor cells were allowed a period of residual synthesis before extraction of the DNA. This interpretation has been criticised by Witkin (1964) on the grounds that UV-inactivation of transforming ability of DNA, and its subsequent recovery, provides a better explanation of the delayed appearance of mutation-transformants.

ii. Tong Ke-zhong, Li Ming-Feng, Mo Hsin-chuan, and Siang Wan-nien (1966), who claimed that mutation to strepto-

mycin-dependence of B. subtilis required posttreatment incubation in the absence of the drug when induced by UV or DES, but not by nitrous acid.

iii. Stewart (1968), who claimed that recombinational mechanisms were not involved in acridine yellow mutagenesis of B. subtilis in the dark. He stated also that acridine yellow at low concentrations would induce reversions only of some spontaneously arising auxotrophs, and not of those induced by UV, nitrous acid, or NTG.

iv. Yoshida and Yuki (1968), who studied NTG-induced reversion of the phe$^-$ marker, and a supposed recovery from NTG-induced lethality. This paper will be examined further in the RESULTS AND DISCUSSION as it is relevant to some data presented here.

At the time of writing, let alone at the commencement of the experiments recorded here, data on the use of B. subtilis in mutation studies was scarce. This paucity is particularly pronounced in regard to the modification of mutation frequencies by ancillary processes. This thesis records the testing of a number of existing auxotrophs of B. subtilis for mutability, the induction, physiological and mutagenic characterisation of many new auxotrophs with a number of mutagens, and the study in depth of the induction of a class of supersuppressor reversion and its modifying factors. These supersuppressors were the first recorded in B. subtilis. Chemical mutagens were predominantly used, especially EMS, but a comparative account of UV mutagenesis is presented. Finally, report is made of the analysis of

an apparent case of "gene-controlled mutational stability" detected during the course of mutation induction experiments.

MATERIALS AND METHODS

1. BACTERIAL STRAINS

Strains of B. subtilis were obtained from the collection of Dr. E.W. Nester (Seattle, Washington, U.S.A.), either directly or via the M.R.C. Microbial Genetics Research Unit. A thymine-requiring strain of SB.168 try-2$^-$ (Farmer and Rothman 1965) was received from Dr. W.D. Donachie. These strains are listed, with their several derivations, in Table 3. Markers have been redesignated according to the recent nomenclature of Dubnau, Goldthwaite, Smith, and Marmur (1967). All strains are derivatives of the classical strain 168, isolated from B. subtilis Marburg by Burkholder and Giles (1947), and discovered by Spizizen (1958) to be capable of genetic transformation.

A large number of auxotrophs were induced during the course of this work, and their details will be found in the RESULTS.

2. MEDIA USED.

i. Sporulation Medium.

From Yoshikawa (1965), containing per litre 8·0 g. Nutrient Broth Powder (Oxoid), 0·25 g. $MgSO_4.7H_2O$., 1·0 g. KCl., 1·25 g. $MnCl_2$., 15 g. Agar (Davis New Zealand or Oxoid Ionagar No.2)., at pH 7·0. After sterilising by autoclaving at 15 lb./sq.in. for 20 minutes, sterile $FeSO_4$ and $Ca(NO_3)_2$ were added, each to 10^{-6} M. The medium was dispensed into sterile vials and allowed to solidify before use in maintenance of cultures. Cultures were grown on this medium for 5 days

TABLE 3. Strains of B. subtilis used in the work described here, other than those constructed in the laboratory.

Strain No. in the Nester Collection	Genetic Markers	Preparative Agent	Parent Strain	Original Reference
SB 19	wild-type	transformation with W 23 threo$^-$ DNA	SB 168 try-2$^-$	Nester & Lederberg 1961
SB 168	try-2$^-$	UV or X rays	Marburg	Burkholder & Giles 1947
SB 1	his-A1$^-$ try-2$^-$	UV	SB 168	Nester & Lederberg 1961
SB 25	his-B2$^-$ try-2$^-$	UV	SB 168	Nester & Lederberg 1961
SB 5	his-A1$^-$ try-2$^-$ ura-1$^-$	UV	SB 1	Ganesan & Lederberg 1964
SB 100	his-B2$^-$ try-2$^-$ tyr-1$^-$	UV and transformation	SB 25	Nester, Schafer, & Lederberg 1963
-	thy$^-$ try-2$^-$	transformation with W 23 thy$^-$ DNA	SB 168	Farmer & Rothman 1965
-	cys$^-$ try-2$^-$	unknown	SB 168	Kelly 1964
-	ad$^-$ try-2$^-$	unknown	SB 168	Kelly 1964
-	arg$^-$ try-2$^-$	unknown	SB 168	Kelly 1964
-	phe$^-$ try-2$^-$	unknown	SB 168	Kelly 1964

at 37'C before long-term storage at 4'C.

ii. Complete Media.

Nutrient Broth (NB) contained 13 g. broth powder (Oxoid) per litre. Nutrient Broth Agar (NBA) contained in addition 15 g. Agar per litre.

Brain Heart Infusion (BHI) contained 37 g. brain heart infusion powder (Oxoid) per litre. Brain Heart Infusion Agar (BHIA) contained in addition 15 g. Agar per litre.

Pennassay Broth (PB) contained 17·5 g. pennassay broth powder (Oxoid) per litre. Thymine (30 µg/ml) was added to all complete media for growth of thymine-auxotrophs.

iii. Minimal Salts Solution.

A five-times concentrated salts solution (Spizizen 1958) contained, per litre, $(NH_4)_2SO_4$ 10 g., K_2HPO_4 70 g., KH_2PO_4 30 g., trisodium citrate 5 g., $MgSO_4$ 1·0 g., this last salt added previously dissolved in a small quantity of water only when all other ingredients were completely dissolved, to avoid the formation of an insoluble precipitate. The salts concentrate was used at 1/5 strength.

iv. Liquid Minimal Medium (MM).

1 litre contained 200 ml. concentrated salts solution, 0·1 ml. 10^{-2} M. $MnCl_2$, and 800 ml. distilled water. It was used undiluted and unsupplemented as diluent, wash fluid, and treatment buffer.

v. Liquid Minimal Growth Medium (MGM).

To a litre of MM were added 27 ml. 20% w/v glucose and 30 µg/ml of each specific growth requirement. For overnight

growth medium, a further 10 ml. per litre 2% w/v casein hydrolysate (Nutritional Biochemicals Corp.) were added.

vi. Transformation Medium 1. (TM-1).

This consisted of MGM modified by halving the $MnCl_2$ concentration, and adding 50 µg/ml of each specific growth requirement and 10 ml 2% w/v casein hydrolysate (per litre).

vii. Transformation Medium 2. (TM-2).

This consisted of MGM modified by total omission of $MnCl_2$, and addition of only 5 µg/ml of each specific growth requirement and 5 ml 2% casein hydrolysate (per litre). Additional $MgSO_4$ was added to 0·01 M.

viii. Solid Minimal Growth Medium (MGA).

MGM without added $MnCl_2$, with 30 µg/ml of each specific growth requirement, doubled glucose concentration (to 54 ml. 20% solution), and 15 g. Agar per litre. Variations of these quantities when used are so described in the text. In some mutation experiments, trace quantities of the specific growth requirements (usually 1·0 - 1·5 µg/ml) or nutrient broth (usually 1·0 - 1·5% v/v) were added to the selective agars. The agar and water, salts, glucose, and required supplements were separately sterilised and mixed only upon cooling to a temperature suitable for plate pouring.

3. MAINTENANCE OF CULTURES.

Stock cultures on sporulation medium were stored at 4°C. If a strain was required for experiment, 2 serial subcultures were made on NBA before the strain was subjected to the rigours of minimal media. This passage on rich medium allowed the germination of spores, an event whose

occurrence on minimal media depends on supplementation. Regular checks were made for the spontaneous reversion of markers.

Stocks used regularly for experiment were maintained on NBA or suitably supplemented MGA at 4'C for periods of 1-3 weeks, when fresh subcultures were made.

4. INCUBATION PROCEDURE.

Liquid cultures were vigorously shaken in a waterbath at 37'C, as were cell suspensions during mutagen treatments. Ehrlenmeyer flasks contained at most a volume of liquid equal to 10% of their capacity to ensure adequate aeration.

Petri plates were incubated at 37'C in the inverted position, unless the plate had been 'spotted' with a drop of mutagen solution. Mutants, plated at 10^{0} and 10^{-1} dilutions, and transformants, plated at 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3}, were incubated for 2-4 days depending on the marker scored; viable counts, plated at the greater dilutions 10^{-4}, 10^{-5}, or 10^{-6}, for 1-2 days dependent on the complexity of the medium used.

5. SCORING PROCEDURE.

Aliquots, usually of 0·1 ml, were spread upon the surfaces of agar plates containing the desired medium. The plates had been dried of excess moisture, either by previous incubation at 37'C, or in a BTL Drying Oven at low mark. In all cases, plates were equilibrated at 37'C in an incubator before experimental plating. The aliquot of inoculum was dispersed over the surface of the plate with a suitably bent

glass rod, previously sterilised by alcohol flaming, and after rapid absorption of the inoculum, incubation was continued at 37°C until colony scoring. In some experiments, where a medial change was desired, spreading and incubation was upon the surfaces of Oxoid 6 cm diameter filter membranes. Membranes were lifted with sterile forceps from medium to medium as and when required.

As it has been suggested (Shields 1963, cited in Loveless 1966) that bacteria treated with alkylating agents may show a sensitivity to spreading, comparative experiments were performed with ethyl methanesulphonate. Mutant yield recovered by the spreading technique was not at all different from that recovered by an "in-plating" technique more usually adopted for bacteriophage work. Spreading was therefore continued.

6. MUTAGEN TREATMENTS.

i. Forward Mutation by UV.

An overnight culture of SB.5 his-A1$^-$ ura-1$^-$ try-2$^-$ in NB was brought to the logarithmic phase of growth in fresh NB, and the cells harvested and washed in MM. UV-irradiation was given to approximately 50% survival, the cells resuspended in NB, and incubation continued overnight. Next morning, the culture was washed in MM, and diluted into a MGM supplemented so as to allow growth only of the parent. After incubation at 37°C for one hour, benzylpenicillin (Glaxo) was added to 200 units per ml, and incubation continued until lysis was evident, usually in 2-2½ hours. The surviving cells were

well washed in MM to remove penicillin, and plated on NBA to recover single colonies. (Membrane filter washing was not a suitable technique, as the flocculate of cell debris prevented flow through the membrane). After incubation for 18-24 hours, colonies were picked with sterile toothpicks on to plates of NBA and of minimal medium so supplemented as to allow growth of the parent. Auxotrophs were detected by their lack of growth on this MGA, and their growth requirements identified where possible by standard auxanographic techniques. UV-induction of auxotrophs was performed once, with the low yield of 5 auxotrophs from 1090 tested colonies.

ii. Forward Mutation by 2AP.

An overnight culture of SB.19 (wild type) in MGM with added casein was diluted into casein-free MGM containing 200 μg/ml 2AP (Sigma Chemical Corp.). Incubation was continued for 4 hours, when the cells were centrifugally washed with MM, and then resuspended with MGM without supplement. After 30 minutes incubation, penicillin selection was performed as described. 9 auxotrophs were recovered from over 2000 colonies tested.

iii. Forward Mutation by EMS.

An overnight culture of SB.19 (wild type) in NB was washed in MM and suspended at 10 times the cell concentration in MM. EMS (Eastman-Kodak), previously dissolved in MM, was then added to a final concentration of 0·1 M. After 30 minutes incubation at 37°C, an equal volume of 6% w/v sodium thiosulphate solution was added, and the cells centrifugally washed three times in MM containing 1% w/v thiosulphate.

Cells were resuspended in fresh NB and incubated overnight. Next day, penicillin selection was performed as described. A second cycle of growth in NB and penicillin selection was also given. As a measure of the efficiency of selection by penicillin, 14 auxotrophs were recovered from 822 colonies tested after the first selection, and 155 from 1003 colonies after the second selection.

iv. Forward Mutation by NTG.

An overnight culture in thymine-supplemented NB of SB.168 try-2$^-$thy$^-$ was diluted into fresh medium and growth continued for 2 hours. Cells were then harvested, washed with MM, and resuspended in MM. A freshly made-up aqueous solution of NTG (Koch-Light) was then added to a final concentration of 25 μg/ml, and the reaction mixture incubated at 37°C for 30 minutes. Three washings in MM were performed with the centrifuge, and the survivors incubated overnight in thymine-supplemented NB. Next morning, the cells were harvested, well washed in MM, and incubated for 5 hours in MGM with tryptophan but no thymine. It was hoped that thymineless death would thus occur in the nonmutant fraction of the cell population, new auxotrophs being protected by their additional requirement (Rolfe 1967). Surviving cells were then plated on MGA with thymine and tryptophan, and 1·5% v/v NB, and after 48 hours incubation, small colonies were tested for auxotrophy. Mutant cells should give rise to small colonies on this plating medium as their new requirement is fulfilled only by the limited quantity present in 1·5% NB. 62 possible auxotrophs were recovered by this means, all being, in fact,

auxotroph. Mutagen treatment was deliberately kept short to reduce the risk of induction of multiple mutants. The recovery technique is modified from Eisenstark et al. (1965).

v. Reverse Mutation by UV.

For nearly all reversion experiments (exceptions are so cited in the text), cells were prepared in MGM with required supplements, and casein hydrolysate for overnight incubation. (See section 9). Next morning, the culture was diluted into fresh MGM with required supplements, but lacking casein, and incubation continued for 3-4 hours. The cells were then in a stage of rapid growth, corresponding to the classical "log. phase". B. subtilis strains derived from SB.168 are sensitive to autolysis if used for posttreatment experiments in the stationary phase, therefore log. phase cultures were used throughout.

UV-induced reversion experiments were carried out in one of two ways:

A. Cells prepared as stated were harvested by centrifugation, washed, and suspended in MM. 3 ml aliquots (2-5 x 10^8 cells per ml) were irradiated in small dishes of 5 cm diameter, giving an effective layer of about 2 mm thickness. Irradiation was performed at room temperature with a Hanovia low pressure mercury germicidal lamp, supposedly with 85% of its output at 253·7 nm in the UV. Distance from source to cell layer was 50 cm, and the dish was manually agitated during the brief exposures, measured in seconds, used to retain high survivals. Accurate dosimetry was not performed as it was deemed unnecessary for these experiments. This technique was used

only in early experiments. Mutants and survival were estimated on the relevant plating media.

B. In subsequent experiments, aliquots of cells (usually to contain 1-2 x 10^8 cells) were impinged upon 6 cm diameter Oxoid filter membranes, which were then held on chilled MGA plates until a number of samples suitable for the experiment had been prepared. The membrane-impinged cells were irradiated, transferred to plates of NBA prewarmed to 37'C, and incubated thus for 2 hours. The membranes were transferred to MGA for 30 minutes to reduce nutrient carry-over from the NBA to the selective medium, and placed upon selectively-supplemented MGA plates for continued incubation at 37'C. Mutants were scored after 2 or 4 days, dependent on the marker studied, and the agar used. B. subtilis yields few UV-induced reversions by the more common direct plating techniques, but this membrane filter method, with 2 hours expression of mutants on NBA allows the handling of larger populations. Points to be noted about this technique (derived from Jensen and Haas 1963) are:

1. That 2 hours on NBA yields the maximal number of mutants for any marker studied. No increase was noted if this incubation was prolonged.

2. Whilst the 30 minute starvation period on MGA did not affect the number of mutants obtained to any detectable degree, it obviously affects background growth of nonmutated cells. Mutant colonies were both larger and easier to score if this starvation period was given. It was shown in specially designed experiments that after a 2-hour period on NBA, mutant

recovery was not diminished by a starvation period of up to $2\frac{1}{2}$ hours before selection on the relevant mutant recovery medium.

In all experiments with UV, irradiation and posttreatments were conducted in dim yellow light to avoid PR. Incubation was subsequently carried out in the dark. PR in B. subtilis was reported by Kelner (1964), although cells competent for transformation were much less capable (if at all) than the cell population as a whole.

vi. Reverse Mutation by EMS.

Cells preparatively grown as stated were resuspended in MM to a concentration of 1-3 x 10^9 per ml, and a quantity of EMS added, previously dissolved in MM. Addition of alkylating agents previously dissolved is recommended by Loveless (1966). In the great majority of experiments described here, a final EMS concentration of 0·075 M was used (0·4 ml EMS in 10 ml MM, added to 30 ml of cell suspension). The mixture was shaken at 37°C in a waterbath for the desired time, then treatment was stopped by the addition of 25 ml. 6% w/v sodium thiosulphate solution. The cells were then centrifugally washed three times in MM containing 1% w/v thiosulphate, resuspended in MM, and either plated or post-treated. Control samples without EMS were always included in experiments.

vii. Reverse Mutation by MMS.

The technique was as for EMS, with the substitution of MMS (Eastman-Kodak) at a final concentration of 0·025 M, as calculated by Strauss (1963).

ix. Reverse Mutation by DEB.

The technique was as for EMS, with the substitution of DEB (Kodak, East Kirkby) at a final concentration of 0·01 M. Thiosulphate cessation of treatment was omitted.

x. Reverse Mutation by 2AP.

An overnight culture of SB.5 his-A1$^-$ ura-1$^-$ try-2$^-$ in MGM with casein was diluted into fresh MGM containing the required supplements and 200 μg/ml of filter-sterilised 2AP. Incubation was continued at 37°C, and at regular intervals samples were removed, washed free of excess 2AP, and plated for mutants and viable count. MGA used for mutant selection contained 1 μg/ml of the previous growth requirement unless the 2AP was given in combination with a UV-treatment, when the protocol (marked B.) for the recovery of UV-induced reversion was followed. 2AP reverted only the ura-1$^-$ marker, mutants forming noticeably smaller colonies than wild-type even after many days incubation.

xi. Reversion Spot-Tests with Mutagens.

Plates of MGA containing 1% v/v NB in place of the growth requirement under test were spread with approximately 10^7 cells from an overnight culture on MGA. A drop of saturated solution of mutagen was then placed in the middle of the plate, and the plate incubated lid upwards at 37°C. Control plates for spontaneous reversion received only a drop of MM. All platings were done in duplicate in 2 independent experiments. 5BU and acridine orange were used only in dim lighting. The test for UV-reversion was performed by irradiation of the cell inoculum on the surface of the plate.

UV irradiation for 20 seconds was given under the conditions previously described. No strong responses to UV were noted, being perhaps a characteristic of the mutant recovery medium used. Reversion tests were scored after 48 hours, thus, hopefully eliminating some partial and suppressor revertants of reduced growth rate relative to wild-type.

Similar modifications of an original technique (Iyer and Szybalski 1958, Szybalski 1958) have been used by Kirchner (1960), Rudner (1961), Balbinder (1962), Allen and Yanofsky (1963), Eisenstark and Rosner (1964), and Cribbs (1965), all with E. coli or S. typhimurium. As the mutagen diffuses through the agar, usually with a zone of killing at the site of application, the small quantity of NB in the medium allows both residual growth of the inoculum to give a larger population of growing cells, and also the expression of induced mutants. The corresponding increase in cell number on control plates allows the determination of very low levels of spontaneous reversion. A comparison of mutant numbers can then be made between mutagen-treated and control plates.

In RESULTS, "marker revertability" has been denoted by the following symbols:

- = no increase in the number of mutants on mutagenised plates in comparison with control plates

± = very small increase possible, although retest on a larger scale would be necessary for a definite result.

+ = small increase on mutagenised plates, up to three times the spontaneous level, or up to 30 colonies per plate where no spontaneous reversion was evident.

++ = good increase on mutagenised plates, 3-10 times the spontaneous level, or 30-100 colonies per plate where no spontaneous reversion evident

+++ = large increase on mutagenised plates, more than 10 times the spontaneous level, or more than 100 colonies per plate where no spontaneous reversion was evident. Most members of this class were immediately recognisable by a confluent ring of revertant growth around the site of mutagen application.

S = no spontaneous revertant ever recovered

rare = spontaneous revertants rare, and not on all plates

R = spontaneously revertible, revertants on every plate.

7. TRANSFORMATION PROCEDURE

i. Competence.

A variety of competency regimens exist for B. subtilis. A modification of the technique of Anagnostopoulos and Spizizen (1961), based on Nester and Lederberg (1961), was used here: The recipient strain was grown overnight in 20 ml NB, and, next morning, centrifuged down, washed in MM, and resuspended to approximately 5×10^7 cells/ml in TM-1. Incubation for 4½ hours in a 37°C waterbath followed, with rapid shaking, then the culture was diluted 1/10 into pre-warmed TM-2, and similarly incubated for another 90 minutes. Transformation was then performed. Each sample tube received 0·3 ml of DNA at the desired concentration, and 2·5 ml of competent cell culture, with incubation and gentle shaking for 30 minutes at 37°C. DNAase (deoxyribonuclease from bovine

pancreas. EC. 3.1.4.5. from B.D.H.) and $MgSO_4$ were added to 20 µg/ml and 0·01 M respectively, and after a further 15 minutes incubation, samples were suitably plated.

Wild type transformants were scored after 2 days incubation at 37°C, and suppressor transformants after 4 days. If the addition of an auxotrophic marker to the recipient was the object of the transformation, samples were diluted into MGM after DNAase treatment and penicillin selected as previously described. The surviving cells were incubated in MGM overnight, the medium suitably supplemented to allow growth of the new class of transformants, and a further penicillin selection performed the next morning, followed by plating. The desired recombinants were always easily recovered.

ii. Preparation of Transforming DNA.

2 methods were adopted, the technique used depending on the purpose of the transformation.

For transfer of genetic markers from one strain to another, a crude preparation of DNA sufficed. The method adopted was that of Jensen and Haas (1963). A 40 ml overnight culture of the donor strain in NB was harvested, washed, and the cells resuspended in 15 ml 0·05 M sodium chloride:0·015 M sodium citrate solution. Lysozyme (crystalline, from egg white, EC. 3.2.1.17, from B.D.H. or Calbiochem.) was added to 50 µg/ml and the mixture incubated at 37°C for 30 minutes with gentle shaking, when an equal volume of 20% w/v sodium chloride solution was added. After a further 30 minutes incubation, 1/20th volume of 2% w/v sodium deoxycholate

solution was added and incubation continued for 60 minutes. The preparation was then stored at 4°C overnight when precipitation of detergent/protein took place. After centrifugation at 5000 g. for 10 minutes to clear this precipitate, the supernatant was added to 2 volumes of ethanol (or 2-ethoxy-ethanol) and the precipitated DNA collected on a glass rod. The DNA was dissolved in 0·015 M sodium chloride solution, and stored at 4°C over a drop of chloroform after an equivolume of 20% w/v sodium chloride solution was added. For marker transfer between auxotrophs, the preparation was used undiluted for transformation.

Where a purer preparation was desired, a much modified Marmur (1961) technique was used. After growth of cells in a complete medium, their harvesting and washing in a solution of 0·15 M sodium chloride solution containing 0·1 M EDTA, the cells were resuspended in this solution at 2-3 g. wet weight of cells per 25 ml. 200 μg/ml lysozyme were added, and incubation carried out at 37°C for 30 minutes with gentle shaking, 2·0 ml 25% w/v sodium lauryl sulphate solution was then added, the mixture held at 60°C in a waterbath for 10 minutes, and then allowed to cool slowly to room temperature. 5 M sodium perchlorate solution was then added to 1·0 M, and the mixture gently shaken. After addition of a equivolume of chloroform: isoamyl alcohol (ratio 24:1) and gentle agitation in a ground glass-stoppered flask for 30 minutes, the preparation was centrifuged at 10,000 g. for 15 minutes. The upper aqueous layer of the three resulting layers was retained, added to 2 volumes of ethanol (or 2-ethoxy-ethanol),

and the precipitated DNA collected on a glass rod. After solution in dilute saline-citrate (DSC), the salt concentration was brought to standard saline-citrate (SSC) by the addition of concentrated saline-citrate (CSC). Concentrated saline-citrate is 1·5 M sodium chloride:0·15 M sodium citrate solution, pH 7·0, and SSC and DSC are respectively 1/10th and 1/100th of this strength. Ribonuclease (from bovine pancreas, EC. 2.7.7.16, from Worthington, previously heated at 80°C to inactivate any contaminating DNAases) was added to 200 μg/ml, with incubation at 37°C for 3 hours, then a further incubation for 3 hours was given after addition of 200 μg/ml Pronase (Streptomyces grisaus protease, from Calbiochem.). The preparation was recycled once from the chloroform:isoamyl alcohol stage after precipitation from ethanol, and the final DNA precipitate redissolved in DSC, with sodium chloride later added to 2 M. The preparation was stored at 4°C over a drop of chloroform.

Before its use for genetical experiments, the preparation was titrated at various dilutions for its transforming ability. In quantitative experiments, a concentration of DNA was used which was not saturating for the recipient cells in the control experiment. DNA concentrations were measured by standard technique (Burton 1956), or by simple conversion from optical density measurements at 260 nm. Biological activity was used as the most important measure of concentration.

8. TRANSDUCTION PROCEDURE.

i. Preparation of Lysates.

Phage PBS1, originally isolated by Takahashi (1961), was supplied by the M.R.C. Microbial Genetics Research Unit. Lysates were prepared by a modification of a protocol suggested by Dr. D. Karamata. In place of Veal Infusion Broth of the original, PB was substituted (Lozeron and Szybalski 1967). A 5 ml culture of the desired donor strain was prepared overnight in PB, and diluted next morning to 1-2 x 10^7 cells/ml in fresh PB. After one hour's incubation with vigorous shaking, PBS1 was added to an estimated 2 x 10^4 particles per ml, and incubation continued for 7-9 hours. The culture was then left standing at 37°C for 2 hours, and at room temperature for the remainder of the night. Next morning, cells were removed by centrifugation, the supernatant treated with DNAase (20 µg/ml for 1 hour at 37°C), and stored at 4°C after sterilisation by membrane filtration.

Many attempts to estimate the total phage titre in these preparations proved unsuccessful, an experience reported by many other workers (e.g. Dubnau et al. 1967), although not always in print. The very recent finding that B. subtilis phage PBS1 may be titrated (-and give visible plaques - the root of the problem -) on Bacillus licheniformis ATCC 8480 (Copeland and Marmur 1968) promises improvement in this respect, although it came too late for this work. Garro and Marmur (personal communication) have shown that the number of transducing particles in a PBS1 lysate is more a function of the method used to prepare the lysate than of the total phage titre.

It therefore seems of little importance in experiments of the kind reported here that the total phage titre be known. As will be seen in RESULTS, the experiments involved comparison of transduction in samples of the same treated bacterial suspension upon different plating media. All phage preparations showed transducing activity.

ii. Transduction.

Recipient cells were prepared as for transformation. At the end of the four-and-a-half-hour incubation in TM-1 however, equal volumes of culture and phage lysate prepared in the desired donor were mixed and incubated at 37°C for 30 minutes. The cells were then collected on a filter membrane, comprehensively washed with MM, resuspended, and plated for transductants. The use of actively growing recipient cultures follows the demonstration by Joys (1965) that phage PBS1 adsorbs only to flagellated B. subtilis strains, such flagellation being a characteristic of rapidly growing cultures.

Control samples were always included, receiving PB instead of phage lysate. The selective agar plates were then incubated at 37°C before scoring of transductant colonies.

9. DEFECTIVE LYSOGENY IN B. SUBTILIS

It has been known for some years past that, like many other bacterial species, strains of B. subtilis harbour a variety of defective phages (Seaman, Tarmy, and Marmur 1964, Ionesco, Ryter, and Schaeffer 1964, Stickler, Tucker, and Kay 1965, Subbaiah, Goldthwaite, and Marmur 1965, Bradley 1965,

Okamoto, Mudd, Mangan, Huang, Subbaiah, and Marmur 1968, Okamoto, Mudd, and Marmur 1968, Haas and Yoshikawa 1969). These phages are induced by a number of agencies, including mitomycin C, nalidixic acid, thymine starvation, ultraviolet light, and other treatments of mutagenic value. Their existence was independently inferred by this author before work on this thesis began from the peculiar behaviour demonstrated by UV-irradiated strains of B. subtilis during post-irradiation growth in rich media, when survival continued to decrease. With the cooperation of Dr. K.W. Jones, phage-like particles were demonstrated with the electron microscope.

A strain of B. subtilis has been isolated from the SB. 168 try-2$^-$ thy$^-$ mutant already referred to which has either been'cured' of these lysogens, or, more probably, rendered noninducible for them. (Karamata 1968, Gross, Karamata, and Hempstead 1968). More widespread use of this mutant and its derivatives should totally remove doubts held by workers about a possible influence of these phages in mutation experiments. The strain was however only released by its discoverer in July 1969.

It is quite evident that a phage induction response to UV, and perhaps other mutagenic treatments, may very well introduce complications to experiments of the kind reported here. These complications, if any, were minimized by two factors:

1. It is well known that the induction of lysogenic bacteria is very strongly dependent upon the medium. Very little, (if any) induction occurred after UV with cells grown in a

minimal medium (e.g. Lwoff, Siminovitch, and Kjeldgaard 1950) in contrast to the massive induction shown by cells grown in a rich medium. Minimal media were therefore used for preparative growth of all strains, except, where cited, for special experiments.

2. Mutagen doses were used which resulted in little lethality. It was felt that under these conditions, any effect of defective lysogens and their induction could be disregarded.

RESULTS

R E S U L T S.

1. Reversion Spot-Tests with known Genetic Markers.

The possession of genetic markers of suitable characteristic is a prerequisite for the performance of mutation experiments. A number of strains of B. subtilis were therefore obtained, and spot-tested for reversion by a number of mutagens. The results of these tests are given in Table 4. Each spot-test was performed on duplicate plates and in two replicate experiments, each with adequate controls. In no case was a difference in kind noted between the two experiments. Tests were scored after 2 days incubation at 37'C, with particular attention paid to the presence of large colony revertants. Although it is not possible to say from large colony size and wild-type growth rate that the back-mutation has been a true reversion, true revertants would however be expected to demonstrate these properties.

Large numbers of revertants were recovered only from the his-A1, his-B2, and ura-1 markers, although they differed in the types of revertant colony produced. Whilst his-A1 revertants formed large colonies with growth rate approximating to wild-type, ura-1 revertants were entirely of small colony type. His-B2 revertants contained a mixture of both colony types. The production of revertant colonies of different sizes has been shown in some instances with enteric bacteria to be due to the induction of true, partial, or suppressor back-mutations (Yura 1956, Howarth 1958, Loveless and Howarth 1959, Stadler and Yanofsky 1959, Smith-Keary 1960, Schwartz 1963, and Eisenstark and Rosner 1964)., although these correlations were not necessarily absolute.

TABLE 4. Reversion Spot-Tests of a number of known genetic markers of B. subtilis by a number of different mutagens. The origin of the markers, where known, is given in Table 3. Techniques were as described in MATERIALS AND METHODS section 6 xi.

Genetic Markers	Spontaneous Mutability	Reversion with tested mutagens: UV.	EMS.	DEB.	NTG.	2AP.	5BU.	AO.	Mn^{++}.
try-2	rare	-	-	-	-	-	-	-	-
his-A1	R	+	+++	++	+++	-	-	-	-
his-B2	R	+	+++	++	++	-	-	-	-
tyr-1	R	+	+	-	+	-	-	-	-
ura-1	R	+	++	+	+	++	++	-	-
thy	R	-	-	-	-	-	+*	-	-
cys	R	+	+	-	+	-	-	-	-
ad	R	-	-	-	-	-	-	-	-
arg	S	-	-	-	-	±	-	-	-
phe	R	+	+	-	+	-	-	-	-

Explanation of symbols: S = stable, no evident spontaneous reversion. R = spontaneous reversion evident. rare = very few spontaneous revertants, not on all plates. - = no induced reversion, +, ++, +++ = increasing numbers of induced revertants, from a small but genuine increase over spontaneous numbers (+) to a large number giving concentric growth of revertants around the site of mutagen application, (+++). ± = retest necessary on a larger scale as the induced mutants were only slightly more frequent than spontaneous revertants. * = growth due to feeding of the auxotroph on the mutagen, not to reversion.

All three markers were reverted by UV and the alkylating agents, the ura-1 marker additionally responding to the base analogues 2AP and 5BU. This apparent marker specificity of the base analogues is further examined in section 10 of RESULTS, with special reference paid to a possible interaction of mutagenic treatments.

No other marker demonstrated useful reversion frequencies. An apparent production of a mass of slow-growing revertants of the thy marker by 5BU was found to be due to the promotion of limited growth of the mutant by the mutagen, a thymine analogue.

With the exception of these three markers, it is apparent that the known genetic markers tested do not offer great scope for mutation experiments. In addition, two of these responsive markers demonstrate the same phenotype, thus effectively ruling out their combination in the same genome for comparative purposes. A programme of isolation and classification of new auxotrophs was therefore carried out.

2. Induction and Classification of New Auxotrophic Markers in strains of B. subtilis.

After induction and recovery of auxotrophs, and the establishment of pure cultures by repeated single colony isolations, each auxotroph was classified according to

a. its particular growth requirement, as determined by standard auxanographic techniques, and

b. a spot-test for reversion. EMS was used as a "representative mutagen" for this purpose, as studies of chemical mutagenesis were among the aims of this work.

a. UV-induced auxotrophs.

1090 tested colonies recovered from mutagenised strain SB5 his-A1$^-$ try-2$^-$ ura-1$^-$ yielded 5 new auxotrophs, whose details are given in Table 5. This was an unexpectedly low recovery of auxotrophs, and it is indeed possible that some, even all, the mutants arose by spontaneous mutation. Alternatively, the penicillin selection technique may not have been executed with full efficiency.

The 5 mutants recovered demonstrate 5 separate phenotypes, presumably resulting from 5 independent mutagenic events. All markers reverted spontaneously at low frequency, but large numbers of EMS-induced revertants were only recovered from the leu marker of auxotroph UV2. Revertant colonies were smaller than those of wild-type even after 4 days incubation. Some EMS-induced revertants of the cys marker of strain UV1 were recovered, but these revertant colonies fed the auxotrophic background, presumably by excretion of the required factor. Scoring of mutants was therefore rendered difficult by this enhanced growth of background.

The symbol "am" given to mutant UV3 denotes an auxotrophy partially satisfied by any of a number of amino acids, including glutamic acid, arginine, proline, and sometimes aspartic acid or glycine. It is possible that this auxotroph, (and the many others of roughly similar phenotype recovered in later isolations) is equivalent to the "amm" mutant more recently described by Dubnau et al. (1967). This "amm" mutant was unable to assimilate the ammonium salts supplied as nitrogen source in minimal medium, although it

TABLE 5. Additional auxotrophies induced with UV in B. subtilis strain SB5 his-A1$^-$ try-2$^-$ ura-1$^-$.

Mutant Code Number	Additional Requirement	Reversion Spot-Tests	
		Spontaneous	EMS-induced
UV1	cys	R	++*
UV2	leu	R	+++
UV3	am**	R	-
UV4	met	R	+
UV5	ilva	R	+

Explanation of symbols:

* = revertants feed background growth, thereby rendering scoring of their numbers difficult.

** = see text for an explanation of this marker symbol.

R = spontaneously revertible,

-, +, ++, +++ = response to induced reversion as previously cited.

would grow on relatively large quantities of such amino acids as glutamic acid. The ability of the "am" mutants isolated in the present work to grow on many different amino acids, and mixtures of amino acids, renders them unsuitable for reverse mutation experiments, particularly when combined with other amino acid auxotrophies. The leu marker of UV2 was subsequently used in a great many experiments.

b. 2AP-induced auxotrophs.

9 auxotrophs were recovered from more than 2000 colonies tested of mutagenised strain SB19 wild-type. Their details are given in Table 6. Although such a small number of auxotrophs could conceivably be of spontaneous origin, a low frequency of induced forward mutation cannot be due to a lack of penetration of the mutagen into B. subtilis for, as will be seen in section 10b of RESULTS, 2AP induces reversion of the ura-1 marker. Alternatively, and as previously suggested, penicillin selection may not have been applied under optimal conditions.

The 9 isolated markers demonstrate 8 different phenotypes. Probably 9 independent mutations are represented as the two markers of threo phenotype show great differences in spontaneous and induced mutability. The leaky his (AP1), leaky leu (AP8), and am (AP6) phenotypes were immediately discarded as unsuitable for reverse mutation studies. All other markers yielded small numbers of spontaneous revertants, and all were induced to revert by EMS. The threo (AP3) and gly (AP9) markers proved more sensitive to EMS-induction of

TABLE 6. **Additional auxotrophies induced with** 2AP in **B. subtilis strain** SB19, **wild-type.**

Mutant Code Number	Auxotrophic Requirement induced	Reversion Spot-Tests	
		Spontaneous	EMS-induced
AP1	his (leaky)	not tested	
AP2	shik	R*	+*
AP3	threo	R	+++*
AP4	threo	R	+
AP5	try	R	+
AP6	am**	R	++*
AP7	ura	R	++*
AP8	leu (leaky)	not tested	
AP9	gly	R*	++*

Explanation of symbols:

* = more than one colony type represented.

** = see text for explanation of marker symbol.

Symbols for reversion as previously used.

reversion than the other 4 markers tested. Both spontaneous and induced revertants of the shik (AP2) and gly (AP9) markers were of more than one colony type.

The threo marker of AP3 has been subsequently used in a number of experiments, particularly those on an apparent effect of genetic background on marker revertibility detailed in RESULTS section 4. The temperature sensitivity of this threo marker was discovered following a fortuitous observation. A second strain, to which the marker had been transferred, grew on threonineless medium at room temperature. Further examination revealed a wild-type phenotype when incubated at 25'C, with reduced growth rate at 28'C and 30'C, and absolute auxotrophy at temperatures above 32'C.

c. EMS-induced auxotrophs.

1825 colonies tested of mutagenised strain SB19 wild-type yielded 169 auxotrophs. After the standard single penicillin selection, 14 auxotrophs were recovered from 822 tested colonies, and a further 155 auxotrophs from 1003 colonies of survivors of a second such antibiotic screening. This illustrates that the single penicillin selection technique previously given for the isolation of UV- and 2AP-induced auxotrophs was probably insufficient, and may be improved by a second such cycle. Details of the newly isolated mutants are given in Table 7. 26 mutants were discarded for mutation studies because of a very high frequency of spontaneous reversion (mutant numbers EMS 2, 12, 23, 26, 28, 36, 38, 40, 64, 71, 76, 77, 91, 93, 97, 98, 101, 108, 112, 115, 121, 125, 133, 136, 140, and 142), although it is

TABLE 7. Auxotrophies induced with EMS in B. subtilis strain SB19, wild-type, arranged according to phenotype.

Auxotrophic Requirement	Mutant Code Number - all prefixed EMS-	Reversion Spot-Tests	
		Spontaneous	EMS-induced
arg	11	R	++
	37	R (many)	not tested
	39 leaky	R	-
	46	R	+++
	75	R	+++
	79	R	+++
	109	R	++
	127	R	+++
	129	S	-
	148	R	+++
	161	S	+
aro, not shik	73 leaky	R	-
asp	66	R (many)	not tested
cys	149	S	±
cys and met	43	S	+
	63	R	+
	124	S	+
glut	126	R	++
his	122	R	+++
	128	R	+
	169	R	++
homoserine	49	R	++
	57	R	++
	59	R	++
	68	R	+
	130	R	+++
	155	R	+
	168	R	+
ileu	69	R	+

TABLE 7 - continued.

Auxotrophic Requirement	Mutant Code Number - all prefixed EMS -	Reversion Spot-Tests	
		Spontaneous	EMS-induced
ilva	14	R	+
	22	R	-
	29	R	-
	41	R	+
	60	R	±
	65	R	+
	70	R	++
	80	R	-
	100	R	+
	103	R	+
	104	R	+
	117	R	+
	119	R	+
	143	S	-
	151	R	+
leu	3	R	++
	7	R	++
	13	R	+++
	20	R	+++
	21	S	+
	27	S	++
	31	S	++
	50	R	++
	55	R	++
	62	R	+
	96	R	+
	106	R	++
	111	S	+++
	116	R	++
	134	R	++
	152	not tested	
	154	R	-
	157	R	+
	164	R	+++

TABLE 7 - continued.

Auxotrophic Requirement	Mutant Code Number - all prefixed EMS -	Reversion Spot-Tests		
		Spontaneous		EMS-induced
met	30	R		++
	47	R		++
	95 leaky		not tested	
	132 leaky		not tested	
	137	R		+
phe	34	R	(very many)	not tested
	160	R		+
ser	51	R		+
	78	R		++
	102	R		+
ser and gly	1	S		+
	6	R		+
	24	R		+
	74	R		-
	123	S		+
	146	R		-
	156	S		++
	159	S		±
shik	131	R		+
threo	8	R		+
	15	R		+
	18	R		++
	52	R		+
	54	S		-
	58	R		+
	61 leaky	R	(v.many)	not tested
	72	R		+
	99	R		+
	105	R		+
	118	R		+
	165	S		+

TABLE 7 - continued.

Auxotrophic Requirement	Mutant Code Number - all prefixed EMS -	Reversion Spot-Tests	
		Spontaneous	EMS-induced
tyr	110	R	+++
	150	R	+
ad	4	R	+
	10	not tested	
	48	R	+
	114	R	++
	138	R	++
ura	94	S	++
vitamin - actual requirement not identified	139	S	±
complex	9 leaky	not tested	
phenotypes	17	R	+
	153	S	+
	163	R	+
	167	R	+

am mutants - reversion not tested - EMS5, 16, 19, 25, 32, 33, 35, 42, 44, 45, 53, 67, 92, 107, 113, 120, 135, 141, 144, 145, 147, 158, 162, and 166.

Symbols are as used in previous tables.

recognised that this may represent such biologically interesting phenomena as genetic instability or the presence of mutator genes. Mutant EMS 56 was discarded because of its great leakiness, and 10 mutants (EMS 81-90) were accidentally misplaced.

The remaining 132 mutants were then tested for their auxotrophic requirements, revealing 23 gross phenotypes, into which the mutants are grouped in Table 7. The am and complex phenotypes were not suitable for mutagenesis experiments, and were not further studied. It is therefore possible that the 103 remaining mutants may have arisen from a maximum of 103 independent mutational events, down to a minimum of 54 such events if all mutants of similar auxotrophic requirement and reversion behaviour are "copies" of the same mutation. On investigation, 4 further mutants were discarded for their considerable spontaneous reversion frequencies, and another 5 due to leakiness. The remaining 92 may be placed into arbitrary classes as follows:

Class	Spontaneous Reversion	EMS-induced Reversion	Number of Mutants
A	-	-	6
B	-	+	12
C	+	-	7
D	+	+	67

2 mutants were overlooked and remain untested. All mutants of classes B and D are suitable for reversion studies with EMS, showing little or no spontaneous reversion and various degrees of induction. There is no apparent

correlation between spontaneous and induced mutability. Mutants stable to spontaneous reversion demonstrate a full range of responses to EMS-induced reversion, whilst, on the other hand, spontaneously reverting mutants may show no response to EMS. Mutagens other than EMS may induce reversion of mutants classes A and C, although this has not been tested. A number of these markers have been used in subsequent experiments.

d. NTG-induced auxotrophs.

62 new auxotrophs were recovered from a mutagenised try-2$^-$ thy$^-$ strain. 5 of these mutants were immediately discarded, 2 on the grounds of extreme leakiness and 3 for spontaneous reversion at very high frequency. Details of the remaining mutants are given in Table 8.

It is perhaps noteworthy that no fewer than 11 of the 57 remaining mutants demonstrate leakiness, as compared to 6/132 EMS-induced auxotrophs. Whether this reflects a property of the mutagen used, NTG, or of the different selection technique practised (thymine starvation instead of penicillin selection) it is not possible to say.

15 gross phenotypes are represented, although the am and complex phenotypes have not been further studied. In view of the known ability of NTG to induce multiple mutations (Adelberg et al. 1965, Eisenstark et al. 1965, Hirota et al. 1968), treatment with inducing mutagen was deliberately kept short in this experiment. It should be noted that of the 8 mutants of complex phenotypes likely to conceal multiple

TABLE 8. Auxotrophies induced with NTG in B. subtilis strain try-2⁻ thy⁻, arranged according to phenotype.

Auxotrophic Requirement added.	Mutant Code Number - all prefixed NTG-	Reversion Spot-Tests	
		Spontaneous	EMS-induced
arg	1	R	++
	5	R	++
	7	R	++
	13	R	++
	19	S	+
	26	S	+++
	35 leaky	S	-
	47	R	+
	53	R	+
aro, including shik	4	R	-
	9	S	+
	33 leaky	S	-
ala	43	S	+
cys or met	21	R	++
	22	R	++
cys and met	45	S	-
gly	60 leaky	not tested	
homoserine	16	R	±
	34	S	-
	59	S	+
ilva	14	R	-
	42	S	-
	61	R	+
met	15	rare	+
	24	S	+
phe	12	R	+
	18	S	-
	49	S	-

TABLE 8 - continued.

Auxotrophic Requirement added.	Mutant Code Number - all prefixed NTG-	Reversion Spot-Tests	
		Spontaneous	EMS-induced
ad	8	R	+
	20	R	+
	25	S	+
	41	R	+
	44	R	+
vitamin- group 1.	6	S	+
	30	S	+
	56	S	-
vitamin - group 2.	10	R	+
	29	S	+
bases - actual requirement not identified	28 leaky	S	-
	36 leaky	S	-
complex phenotypes	3	S	+
	11	R	+
	23	S	-
	31	S	+++
	39	R (many)	not tested
	50	R	+
	57 leaky	S	-
	58	R	+++
am mutants	17	R	+
	27	R	++
	37 leaky	S	-
	38 leaky	S	-
	40	S	+
	46 leaky	S	-
	48 leaky	not tested	
	54 leaky	R	+
	62	S	-

Symbols are as used in previous tables.
Vitamin requirers may be auxotrophic for
Group 1 - rib., p.a.b.a., nic., or pyridoxine.
Group 2 - inosinic acid, bio., thia., or pan.
Their actual requirements have not been tested.

mutations, five yielded large-colony revertants. At best therefore, it would appear that 3/57 mutants may be multiple auxotrophs, although one cannot rule out the presence of other non-exacting mutations not screened for, or that single auxotrophs contain multiple mutations (e.g. Class A below).

1 mutant was not further examined due to its high spontaneous reversion rate, and 2 mutants on the grounds of leakiness. The remaining 54 mutants may be arbitrarily placed in classes as follows:

Class	Spontaneous Reversion	EMS-induced Reversion	Number of Mutants
A	-	-	16
B	-	+	13
C	+	-	3
D	+	+	22

EMS- and NTG—induction of auxotrophs of B. subtilis are compared, and contrasted with the findings of other workers with other organisms in the DISCUSSION.

All mutants of classes B and D are suitable for reversion studies with EMS, although it is possible that classes A and C may be induced to revert by other mutagens. As with EMS-induced auxotrophs, there is no correlation between the spontaneous and induced revertibility of markers.

3. Comparison of Spot-Test Reversion and more Quantitative Experiments.

Newly isolated auxotrophs have been classified by their degree of EMS-induced reversion in spot-tests into 5

classes, as defined in MATERIALS AND METHODS, section 6 xi. These classes are however arbitrarily determined, and despite the excellent agreement between the responses given by markers in the two replicate spot-tests performed, the validity of the classification has not been proven.

Quantitative experiments have subsequently been performed with a number of these new mutants of the classes likely to be used in future mutagenesis studies. A comparison may therefore be made between the spot-tests and these more quantitative experiments, as shown in Table 9 for 2 such mutant classes. Two separate quantitative experiments were performed with each mutant, and the highest reversion frequency noted in either experiment at a given survival are shown in the last two columns of the Table. Each figure derives from EMS-dose-response experiments at points where mutation induction curves are flattening out to plateau values. In more practical terms, they represent the results of 40-60 minutes treatment with 0·075 M. EMS at 37°C, thiosulphate washing of cells, and their plating on a selective medium optimal for mutant recovery.

Although the 14 mutants examined in this way will be seen to vary in their induced frequencies of reversion within the two arbitrary classes tested, this classification is justified by the great difference shown between classes. This is usually about 100-fold, although as low as 20-fold for mutant EMS 78. Among the 14 tested mutants, there are no exceptions to this correlation between spot-test and quantitative reversion estimates.

TABLE 9. Correlation of Classification by Mutagen Spot-Test and Quantitative Estimation of the Revertibility of some representative alleles of the two most responsive Classes.

Number of Tested Allele	Auxotrophic Requirement	EMS-Spot-Test Classification	Quantitative Estimation of Revertibility by EMS Mutants/10^7 survivors (approx)	approx. survival
AP3	threo	+++	400	75%
EMS110	tyr	+++	400	90%
UV2	leu	+++	290	85%
AP4	threo	++	1·27	60%
EMS6	ser + gly	++	6·9	70%
EMS24	ser + gly	++	3·5	70%
EMS47	met	++	7·8	65%
EMS51	ser + gly	++	0·5	70%
EMS78	ser + gly	++	15·0	65%
EMS100	ilva	++	0·9	100%
EMS118	threo	++	1·79	65%
EMS131	shik	++	1·15	60%
EMS137	met	++	2·14	70%
EMS138	ad	++	3·6	100%

It is surprising to find in the literature that only in the original studies of Iyer and Szybalski (1958) and Szybalski (1958) has a similar correlation been sought between spot-tests and more quantitative experiments. Although Iyer and Szybalski's results showed a good overall correlation between the two techniques, it must be remarked that their experiments involved the reversion of a single locus (streptomycin-dependence to -independence) induced by a multiplicity of mutagenic agents. All other workers have tested a variety of alleles of one or more genes against a few mutagens. No reported attempts have been made to verify their classification by more quantitative experiments with a representative sample of alleles. Although there are no grounds for belief in a lack of such correlation, certainly on the evidence presented here, the extended application of a test devised for one particular system to further unrelated systems surely demands more care. A case in point is the variable behaviour demonstrated by formaldehyde, which is apparently mutagenic only in dose-effect-curve experiments, and not at all (or very weakly so) in spot-tests (Szybalski 1958).

4. Reversion Studies with the his-Al marker in a number of genetic backgrounds, and the discovery of its super-suppressibility.

Influences of genetic background on allele mutability are well known (see Table 1), taking the form of the abolition, reduction, or enhancement of induced mutation, or of the

alteration of induction kinetics. The his-A1 marker of B. subtilis has been shown to be revertible by a number of mutagenic agents (Table 4), and it was of interest to compare its mutability in a number of genetic backgrounds. Alteration of genetic background was performed by the addition of new markers, either directly by mutation, or indirectly by transformation with DNA isolated from a strain bearing a desired additional mutation.

Three responses of the his-A1 marker were studied - its spontaneous, UV-induced, and EMS-induced reversion.

a. Spontaneous mutability of the his-A1 marker shows an independence of initial plating density on agar supplemented with traces of histidine or NB. This has been previously reported for the try-6 marker of E. coli B/r WP2 by a number of workers (e.g. Demerec and Cahn 1953, Deering 1963, Chopra 1967). Table 10 records the yield of spontaneous his-A1^{+} revertants recovered over a wide range of initial plating densities in a typical experiment. That these mutants arose on the 1% v/v broth-enriched plating medium during periods of residual division is illustrated by the very small numbers of such revertants obtained on selective agar without broth, and only at high plating density.

It is evident that mutants already present at the time of plating do not form an important proportion of recovered revertants, either in the his-A1 marker studied here, or in the try-6 marker of E. coli studied by Chopra (1967) where similar findings were reported. Chopra also demonstrated a selection against these reversions in previous growth in

TABLE 10. Numbers of spontaneous revertants of the his-A1 marker recovered after plating at different plating densities on agar with or without 1% v/v NB. Each figure is the mean count of three plates. The strain used was UV2 his-A1$^-$ try-2$^-$ ura-1$^-$ leu$^-$.

Number of cells inoculated per plate, calculated from one viable count and the dilution factor.	Histidine revertants per mean of 3 plates, on selective agar	
	WITHOUT BROTH	WITH BROTH
$7{\cdot}95 \times 10^6$	4	125
$3{\cdot}975 \times 10^6$	2	88
$1{\cdot}59 \times 10^6$	0	87
$7{\cdot}95 \times 10^5$	0	100
$3{\cdot}975 \times 10^5$	0	99
$1{\cdot}59 \times 10^5$	0	75
$7{\cdot}95 \times 10^4$	0	82
$3{\cdot}975 \times 10^4$	0	63
$1{\cdot}59 \times 10^4$	0	54
$7{\cdot}95 \times 10^3$	0	39
$3{\cdot}975 \times 10^3$	0	36
$1{\cdot}59 \times 10^3$	0	21

liquid medium. As with this classical system in E. coli, it would appear that a standard trace-supplemented agar is able to support residual division of an inoculum until a maximal cell population is reached. The size of this cell population is a characteristic of the medium, not of the inoculum size. As spontaneous mutants will arise in proportion to this cell population, approximately equal numbers of mutants are recovered per plate over a wide range of plating densities. Reduction in mutant numbers at the lower plating densities may be explained by the permission of more residual divisions per cell plated at low densities by the medial supplement than to corresponding cells in a larger inoculum. This may result in the more frequent occurrence of more than one revertant per microcolony.

Tests for spontaneous reversion of markers are thus simple to perform on trace-supplemented medium, as the increase in size of the studied population by residual division allows the detection even of very rare revertants. Interference with the production of revertants may be easily detected as a decrease (or increase) in mutant number, and simple analysis should reveal whether genotypic or phenotypic causes are responsible.

The results of such tests with the his-Al marker are given in Table 11. As large effects were sought, rather than small alterations in frequency of spontaneous mutation of possible trivial explanation, results are expressed in symbols rather than in figures. It will be seen that of the 8 markers tested for an effect on histidine reversion,

TABLE 11. Spontaneous and Induced Reversion of the his-Al marker in a number of genetic backgrounds.

Genotype of Strain	New Marker	HISTIDINE-REVERSIONS			REVERSIONS OF NEW MARKER		
		Spont-aneous	UV-induced	EMS-induced	Spont-aneous	UV-induced	EMS-induced
his-Al	his-Al	R	++	+++	-	-	-
his-Al try-2	try-2	R	++	+++	rare	-	-
his-Al try-2 ura-1	ura-1	R	++	+++	R	+	++
his-Al try-2 ura-1 leu	leu (UV2)	R	++	+++	R	++	+++
his-Al try-2 ura-1 leu threo	threo (AP3)	rare	++	+++	R	++	+++
his-Al try-2 ura-1 leu threo^{+}	threo^{+}(spont.)	R	++	+++	-	-	-
his-Al try-2 ura-1 cys	cys (UV1)	R	++	+++	R	+	++
his-Al try-2 ura-1 arg	arg	R	++	+++	S	-	-
his-Al try-2 ura-1 met	met (UV4)	R	++	+++	R	+	+
his-Al try-2 ura-1 ilva	ilva (UV5)	R	++	+++	R	+	+

Symbols used are as previously described.

only the threo (AP3) marker produced any effect. Very few, if any, histidine revertants were recovered from a threonineless background, in contrast to the 50-150 colonies per plate usually recovered from the other strains, varying by experiment. Restoration of the threo$^+$ phenotype by spontaneous mutation returns the histidine reversion characteristic normally associated with the his-A1 marker. At first sight, this specific effect of the threonineless mutation on spontaneous histidine reversion is strikingly reminiscent of the phenomenon of "gene-controlled mutational stability" reported by Chopra (1967) in the WP2 system of E. coli. The threonineless effect is analysed in detail in section 12 of RESULTS.

b. UV-induced reversion of the his-A1 marker was studied by the high-yield filter membrane technique described in MATERIALS AND METHODS, section 6.v.B. Although small variations were recorded in individual experiments, none of the 8 markers tested produced any large and repeatable effect on UV-induced histidine reversion.

c. EMS-induced reversion of the his-A1 marker was similarly unaffected by any other marker. It is noteworthy that the threo (AP3) marker which produced an effect on spontaneous histidine reversion had no such effect on induced reversion.

In all these induction experiments, the response of the newly added marker was studied with respect to spontaneous and induced reversion at the same time as that of the his-A1 marker. The induction of revertants of leu (UV2)

proved of some interest, as in some experiments a close correlation was noted between the numbers of induced histidine and leucine revertants.

5. Supersuppression of the his-A1 and leu (UV2) markers.

The close correlation observed between numbers of histidine and leucine revertants observed in some experiments prompted the examination of these revertants for super-suppression, or "joint relief", of the two markers.

a. Spontaneous Revertants.

Spontaneous revertants of both markers were collected and streaked with sterile toothpicks on to the relevant medium to test for joint relief of the second marker. All mutants isolated as leucine revertants on selective medium, supplemented or not with traces of leucine or NB, proved to be jointly independent of histidine. These spontaneous leu^+ mutants, which are not very frequent in occurrence, are therefore $su^+_{leu,\ his\text{-}A1}$ mutants.

In contrast, only a small fraction of histidine revertants had simultaneously become independent of leucine. Given in Table 12 are the results of a number of experiments where histidine revertants, isolated on selective agar with traces of histidine or NB, have been tested for coindependence of leucine. Only 15% of these his^+ revertants overall were leucine-independent, signifying that most revertants of the his-A1 marker are either true or partial back-mutations, or suppressor mutations affecting the his-A1 marker and not the leu marker.

TABLE 12. Test of spontaneous revertants of the his-A1 marker for simultaneous acquisition of leucine independence.

Experiment number.	Recovered on selective agar + traces of:	Number of his^+ revertants tested	his^+ leu^- phenotype	his^+ leu^+ phenotype
1	histidine	18	14 (78%)	4 (22%)
2	histidine	56	54 (96%)	2 (4%)
3	NB	61	57 (93%)	4 (7%)
4	NB	112	96 (86%)	16 (14%)
5	histidine	264	237 (90%)	27 (10%)
6	NB	281	216 (77%)	65 (23%)
TOTAL TESTED		792	674	118
		100%	85%	15%

Growth of the suppressor-containing mutants was similar to wild-type on selective medium containing normal quantities of leucine, but much reduced on selective medium without supplement or with normal quantities of histidine. This probably reflects the efficiency of suppression of each marker.

b. Induced Revertants.

Mutation induction experiments with both UV and EMS demonstrated that most, if not all, induced reversions of the his-A1 and leu markers are in fact supersuppressor mutants.

Table 13a records one of three UV-induction experiments performed. Other than the application of different UV doses, the technical conditions of the experiment involved the identical handling of replicate filter-membrane-borne cell populations. All replicate populations received after UV a two-hour incubation period on NBA, and the revertant numbers therefore represent the ability of induced, fixed, and expressed mutants to grow on the three different selective media. It will be seen that there is near-equality between the numbers of mutants of each type selected.

Table 13b records the analysis of EMS-induced revertants by a different approach. Colonies of histidine or leucine revertants were streaked with sterile toothpicks on to the surface of a further selective medium to detect the simultaneous relief of the second auxotrophy. It will be seen that almost all leu^+ revertants are also his^+: the 4 exceptions may be due to manual error while streaking, although this has not been tested. 90% of his^+ revertants are also

TABLE 13. Induced Supersuppression of the his-A1 and leu markers.

a. UV-induced revertants isolated on different selective media from aliquots of irradiated cells.

UV-dose in seconds	Induced mutants per mean of three membranes, after subtraction of spontaneous revertants, selected as		
	HIS^+	LEU^+	HIS^+ LEU^+
5	161	145	148
10	403	382	371
20	883	903	854

b. EMS-induced revertants of the his-A1 and leu markers, tested for relief of the second marker.

Experiment number	Original Isolation as	Number Tested	Second Marker Relief in
1	leu^+	54	52
2	leu^+	193	193
3	leu^+	108	107
4	leu^+	61	60
5	his^+	31	27
6	his^+	77	76
7	his^+	50	42
8	his^+	100	89

leu$^+$, the exceptional 10% in all probability representing the predominantly his$^+$ leu$^-$ mutants of the medial-dependent spontaneous background.

c. Analysis of Supersuppression.

Supersuppression was verified by two genetical means:

1. In the first test, 3 independently isolated supersuppressor revertants of the his-A1 and leu markers were transformed with DNA from the wild-type strain SB19. Search was then made for transformants to su$^-$, once again requiring histidine and leucine. As these su$^-$ transformants had to be recovered by penicillin selection in the manner of new auxotrophs, this technique is of necessity qualitative. Doubly requiring histidine and leucine clones were recovered from all th ee revertants tested, however, demonstrating that they were truly supersuppressed.

2. In the second test, the su$^+$ mutation was transferred to new cultures of the parent histidine and leucine auxotroph to look for joint relief of these requirements. Table 14 shows that supersuppression of the his-A1 and leu markers was transferred at approximately the same frequency as a reference try$^+$ marker, whether via preparations of transforming DNA or by the transducing phage PBS1. Coincident transfer of the wild-type alleles of the studied markers occurred at a much lower frequency, even when saturating concentrations of transforming DNA were used.

An additional piece of information may be derived from these experiments. The lack of cotransformation or cotransduction of the his-A1 and leu markers demonstrates that they

TABLE 14. Genetic Transfer of the su^+ character by transformation and transduction.

a. TRANSFORMATION.

Experiment.	Donor DNA.	Transformants per aliquot of DNA treated competent cell culture.		
		his^+ leu^+ doubles.	su^+	try^+
1	wild-type	1*	-	60
2	wild-type	27*	-	1820
3	wild-type	56*	-	1080
4	su^+	-	505	692
5	su^+	-	336	601
6	su^+	-	32	59

* = saturating DNA concentrations used.

b. TRANSDUCTION WITH PHAGE PBS1.

Experiment	Donor strain	Transductants per ml. recovered as		
		his^+ leu^+ doubles	su^+	try^+
1	wild-type	1	-	1340
2	wild-type	0	-	760
3	wild-type	0	-	2500
4	wild-type	0	-	290
5	su^+	-	410	990
6	su^+	-	290	830

are not part of the same linkage group. The position of the his-A1 marker on the genetic map of B. subtilis is known (Dubnau et al. 1967), falling in group II of the four known linkage groups. The leu marker induced and used in the present work is obviously not in the same linkage group, although it is not known whether it is part of linkage group III with the leu-1 and leu-2 markers described by Dubnau et al. (1967).

d. Range of Supersuppression.

Joint supersuppression of up to 5 separate mutations has been demonstrated in a single strain of S. cerevisiae (Mortimer and Gilmore 1968). It is likely therefore that supersuppressor mutations relieving the his-A1 and leu markers of strain UV2 of B. subtilis will also affect other markers. 5 of the newly induced auxotrophies were examined for this phenomenon, all previously characterised by a high frequency response to EMS-induced reversion in spot-tests. The mutants tested were threo (AP3), ser-gly (EMS78), tyr (EMS110), his (EMS122), and homoserine (EMS130). 2 tests were applied:

1. Strains monoauxotrophic for the markers to be tested were transformed with DNA preparations either of strain SB19 wild-type or of a su^+ revertant of strain UV2. As su^+ revertants had been shown to demonstrate slower growth than wild-type on some media, it seemed possible that suppression of a third marker might be detected by a comparison of prototrophic transformant colonies of that marker from both types of DNA preparation. Whereas wild-type DNA can only supply wild-type

alleles, su^+ DNA may obviously supply both wild-type and suppressor genes in this particular situation. Search was made for the presence of two colony types among transformants to prototrophy of the five markers with su^+ DNA, in comparison with wild-type DNA. None of the 5 tested strains showed two such colony types. This result does not of course prove that the 5 tested markers could not be supersuppressed with the his-A1 and leu markers, only that if they were, su^+ colonies showed wild-type growth rate in these strains. A negative result in this kind of presumptive test is obviously valueless.

2. Derivatives of strain UV2 (his-A1$^-$ leu$^-$ ura-1$^-$ try-2$^-$) were prepared by transformation, each additionally carrying one of the 5 markers under investigation. Each derivative was then tested for spontaneous and EMS-induced supersuppression of the his-A1 and leu markers as before, and, in addition, the ability of these supersuppressors to relieve the new requirement. (As the his (EMS122) marker could obviously not be tested in the presence of the his-A1 requirement, strain UV2 was transformed to his-A1$^+$ before addition of the his (EMS122) marker. The leu and new his markers were then tested for cosuppression).

None of the 5 tested auxotrophies were jointly supersuppressible with the his-A1 and leu markers. Evidently revertants of these 5 markers represent mutations other than the supersuppressors described, although it remains quite possible that they constitute or contain other classes of suppressor mutations not affecting the his-A1 and leu markers.

In studying their revertants and su^+ reversion in the same strain, we are thus not measuring the same mutation on two different plating media.

Summary of supersuppression to date: supersuppressor mutants have been isolated in B subtilis giving joint relief of the his-A1 and leu mutations. Tests have shown that about 15% of spontaneous his^+ revertants, 100% of spontaneous leu revertants, and the great majority of induced revertants of both markers are supersuppressor revertants. Genetical verification of supersuppression has been made by

a. recovering the original pair of auxotrophies from revertants after a cross with wild-type DNA, and

b. demonstration that the su^+ character may be transformed at approximately the same frequency as other single mutations, giving joint relief of the his-A1 and leu markers in the recipient strain. None of 5 other tested markers were relieved by these supersuppressors.

This discovery of supersuppression in B. subtilis* constitutes the first report of the phenomenon in a Gram-positive microorganism, although allele-specific suppression has been previously reported in E. coli, S. typhimurium, S. cerevisiae, and S. pombe. More recent reports of supersuppression in B. subtilis have been offered by Georgopoulos (1968, 1969) and Okubo and Yanagida (1968).

It seemed likely that the discovery of readily inducible

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* reported in Microbial Genetics Bulletin 1967, No. 26, pp. 5-6.

supersuppressor mutations might be a valuable one. In 1963, Witkin reported in an abstract that about one third of the auxotrophs isolated from strains B/r and K12 of E. coli were reverted at high frequency by UV. Of greater interest, however, was her demonstration that induced mutation frequency of these markers, many of them supersuppressible, was drastically altered when postirradiation protein synthesis was inhibited. Interest in a possibly comparable behaviour of supersuppressor mutations in B. subtilis, induced by UV or chemical mutagens, was heightened by the description of the suppressor-specific effects of certain ancillary conditions in mutant WP2 of E. coli B/r (Bridges, Dennis, and Munson 1967). These ancillary conditions apparently failed to modify induced mutation frequency of nonsuppressor revertants in a similar manner. A detailed study was therefore commenced of the induction of supersuppressor mutants in B. subtilis, with especial reference paid to the modification of mutation frequencies by ancillary conditions. Possible sources of influence studied include the nutritional status of the organism, both before and after treatment, such physical conditions as temperature and solid or liquid phase of incubation, and the effects of various posttreatments.

6. The Induction of Supersuppressor Mutants of B. subtilis with EMS.

a. Mutation Induction and Dose Response.

Supersuppressor revertants were readily induced in strain UV2 his-A1$^-$ leu$^-$ ura-1$^-$ try-2$^-$ with EMS, giving joint relief of the his-A1 and leu markers. When cells were grown

to log. phase in suitably supplemented minimal medium, harvested, EMS-treated, and plated for mutant recovery, supersuppressor yield increased with the length of mutagen treatment. Table 51 presents the results of such a dose response experiment using 0·075 M. EMS. Survivals at the various doses are not given, as they represent the effects not only of mutagen-induced lethality, but also of any cell loss during the centrifugal washing procedures practised. As a matter of policy (MATERIALS AND METHODS section 9), high survivals were both required and obtained. Survival at any dose was very similar on the three media used to score it, although counts were sometimes fractionally higher on selective agar supplemented with traces of broth. In the experiment shown in Table 15, observed "survival" after 70 minutes of EMS treatment, the highest dose, scored on selective agar plus 1·5% v/v NB, was 57·6%. The interesting comparisons in this and similar experiments lie not between doses, however, but between mutant recoveries on different media at each dose.

Mutation yield at each dose was increased if trace quantities of the previous growth requirements were included in the selective agar. The magnitude of this stimulatory effect decreased with increasing EMS dose. An even greater increase was given by incorporation of traces of NB into the selective agar: this extra enhancement compared to that of histidine and leucine being subsequently termed the "broth effect". The magnitude of this broth effect lay between three- and four-fold, although this level was not attained at the lowest doses of EMS in contrast to the stimulation by

TABLE 15. The Induction of Supersuppressor Mutants with EMS and their Recovery on Media of Different Supplementation.

No.I.

Mutants recovered on selective agar Plus:	Time of Exposure of Cells to EMS (0·075 M., 37°C) in minutes:						
	10	20	30	40	50	60	70
NO ADDITION	0·105 10	2·4 220	8·0 480	13·6 1060	33·8 1910	52·0 3110	- -
1·5 µg/ml EACH OF HISTIDINE AND LEUCINE	1·2 120	11·8 1100	49·1 3240	81·1 5840	124·6 7100	194·7 11100	235·9 16750
1·5% v/v NB	2·0 220	39·8 4450	179·5 14900	257·7 25000	476·0 37600	697·9 49550	741·0 53350
BROTH EFFECT	1·66	3·37	3·66	3·18	3·82	3·58	3·14

Of the paired figures given for each dose and medium, the upper figure is the number of mutants induced per 10^7 survivors. Mutants and survivors were scored on the same type of agar, except where mutants were scored on unsupplemented selective agar, when the viable count was scored on fully supplemented minimal agar. Selective agar was MGA plus 30 µg/ml each of uracil and tryptophan. The lower figure in each pair is the actual number of mutants per ml. of suspension plated, calculated from the dilution factor and plate counts, and corrected for any spontaneous mutants. The "Broth Effect" is the mutation frequency on broth-supplemented agar divided by that at the same dose on histidine and leucine-supplemented agar.

TABLE 16. The Induction of Supersuppressor Mutants with EMS and their recovery on different media.

No. II.

Mutants recovered on selective agar plus:	Time of exposure of cells to EMS (0·075 M, 37°C) in minutes					
	10	20	30	40	50	60
1·5 μg/ml each of HISTIDINE and LEUCINE	1·5 70	11·6 640	55·1 3120	81·2 3900	131·4 2800	135·9 12500
1·5% v/v NB	2·6 250	32·2 2990	146·1 11400	248·9 22400	466·0 21900	495·2 52000
"Broth Effect"	1·75	2·76	2·65	3·06	3·55	3·64

The legend to this Table is the same as that of Table 15.

histidine and leucine. Table 16 presents the results of a similar experiment designed to reexamine the broth effect. Once again, as in many other experiments, its magnitude lay between three and four fold, although lower at the smaller doses of mutagen. Similar phenomena have been reported in an E. coli system for UV-induced reversion (Clarke 1967).

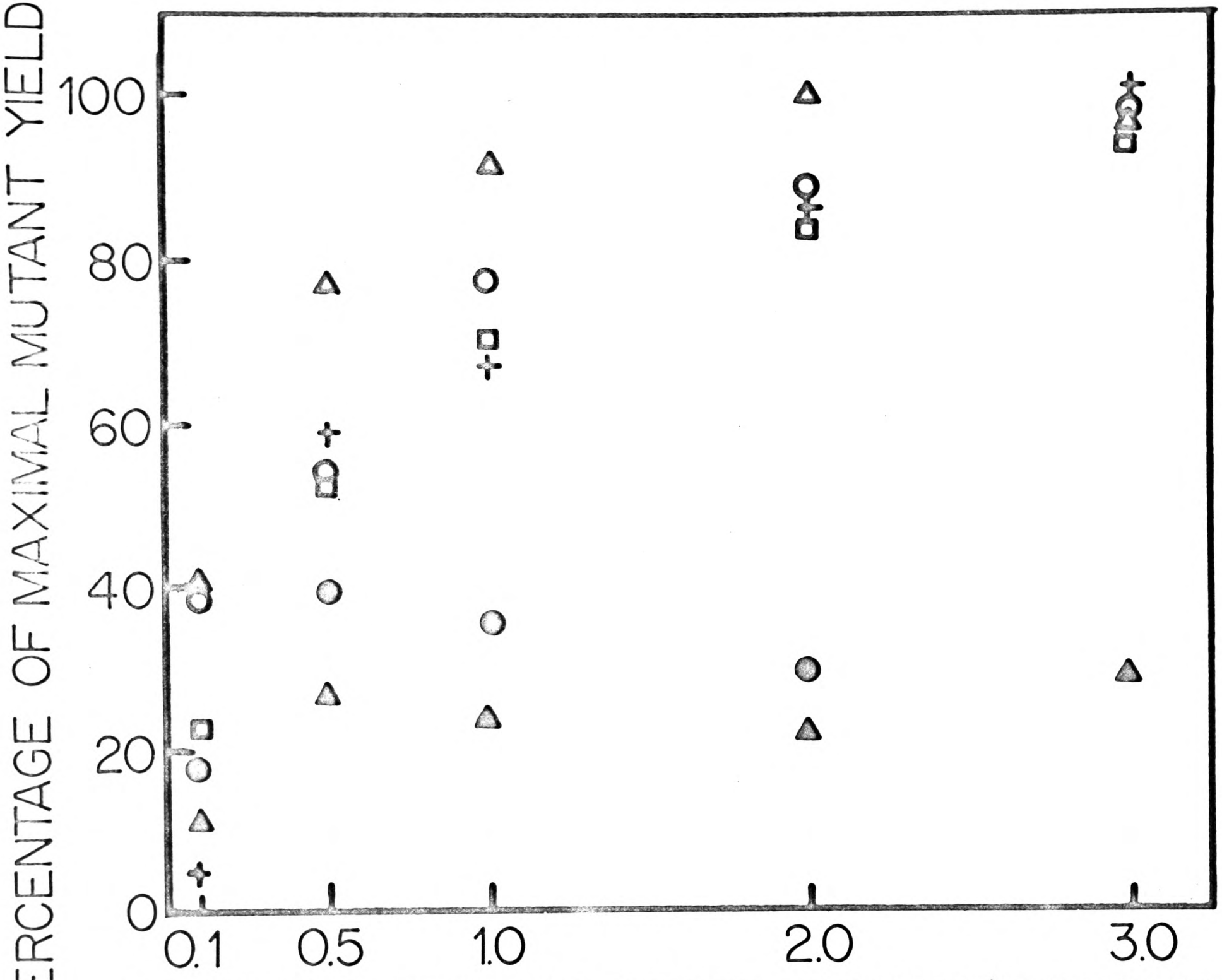
The enhancement of mutation yields by trace supplementation of the selective agars, and especially the superiority of broth in this respect, poses a number of questions. Is this extra enhancement by broth due simply to the presence in broth of more histidine and leucine than is added to trace-amino acid-supplemented plates alone? Alternatively, are components of the broth other than histidine and leucine responsible? And, if a genuine additional enhancement by broth occurs which is not explained away by mere differences in supplement concentration, in what manner and over what period does broth exert this effect?

b. The Effect of Broth Supplementation.

The relationship between the stimulations of mutant yield shown both by broth and by histidine and leucine has been examined by titrating the effects of various concentrations of these supplements. Samples of cells, grown to log. phase in suitably supplemented minimal medium, were given a single dose of EMS. Aliquots of washed cells were then plated out on mutant recovery media containing various amounts of the two kinds of enhancing supplement. The results of 4 separate experiments with broth and two with histidine and leucine are combined in Figure 1. For purposes

FIGURE 1.

Dependence of mutation yield on trace supplementation of the selective agar. Individual experiments are represented by different symbols. Filled symbols = plated on selective agar with added histidine and leucine, open symbols and crosses = plated on selective agar with added broth. Mutation yield is derived from the number of mutants arising from aliquots of cells receiving uniform EMS doses in each experiment.


PERCENTAGE OF MAXIMAL MUTANT YIELD
100
80
60
40
20
0
0.1
0.5
1.0
2.0
3.0
CONCENTRATION OF ADDED NUTRIENT BROTH
(IN % v/v) OR HISTIDINE AND LEUCINE
(µgm/ml OF EACH)

of comparison, mutant yields have been expressed in percentages of the maximum recovered in each experiment. Whilst there is a little variation between the experiments, it is clear that the maximum effect of supplementation with histidine and leucine occurred at a concentration of 0·5 µg/ml of each amino acid. No further increase in mutation yield was given by four to six times these amounts. In contrast, broth supplementation gave a larger and more rapid increase in mutation yield than the two amino acids, continuing up to the highest levels obtained. From these experiments, it is evident that the 1·5 µg/ml each of histidine and leucine used in the preliminary dose effect experiments was not a limiting factor. The extra enhancement shown by broth is therefore due to components of the broth other than histidine and leucine. Similar experiments have been performed with a mixture of casein hydrolysate and tryptophan (added because preparation of casein by acid hydrolysis destroys its tryptophan content). The results mimicked those of broth, demonstrating that the extra stimulation shown by broth is due to components of its amino acid pool.

Concentrations of supplements greater than those given in Figure 1 were also used in the same experiments. With a further increase in supplementation, greater background growth occurred of the nonmutant cells, forming a thick lawn of growth on the plate at concentrations over 3% v/v NB or 4-5 µg/ml of both histidine and leucine. This increased background growth had undesirable effects upon mutant colonies, which were diminished in size and in

consequence more difficult to score. This did not become a serious problem, however, as the optimal supplementation for mutant recovery lay between 2-3% v/v NB or 0·5-1·0 μg/ml each of histidine and leucine for the two types of agar. Increasing NB concentrations up to 5% v/v gave no further increase in mutant yield. In the great majority of experiments to follow, scoring of mutant colonies was facilitated by limiting the concentrations of supplement added to the 1·5% v/v NB or 1·5 μg/ml each of histidine and leucine originally (and fortuitously) used. This allows recovery of about 80-90% and 100% respectively of mutants recoverable on the two types of media.

Two other possible explanations of the broth effect have been eliminated, in so far as is possible. Firstly, differences in relative survival were not responsible for the various degrees of stimulation of mutant recovery shown. Survival was scored in these same experiments on the types of agar used for mutant recovery. Agars containing 0·1-5% v/v NB or 0·5-5·0 μg/ml each of histidine and leucine showed no appreciable differences in survival counts. (Colonies of survivors scored on agar with 0·1% v/v NB were very small and were scored with the aid of a low powered dissecting microscope). It should be noted, however, that there is a possible error inherent with all reconstruction experiments of this kind. Survival measured at the great dilutions normally used cannot be shown to be the same as survival at the densities at which cells are plated for mutant recovery.

Secondly, mutants arising in response to trace supplementation of the recovery medium were not of a different

phenotype, perhaps requiring the trace supplement for growth. Of several hundred revertants obtained on trace-supplemented agars and restreaked on unsupplemented mutant recovery agar, not one proved incapable of growth. The effect of medial supplementation is obviously upon mutation expression.

c. MFD on Solid Media.

Similar broth effects have been detected in UV-mutagenesis with strains of S. typhimurium and E. coli (Witkin 1956, Munson and Bridges 1966, and Clarke 1967). Similarly to what has been shown here for B. subtilis, the results of Witkin and Clarke demonstrate that the broth effect can be mimicked by a nonspecific pool of amino acids (or casein hydrolysate). Increase in UV-induced mutation frequency has also been shown in E. coli by Doudney and Haas (1958) after a preplating posttreatment in amino acid-rich liquid medium.

The temporal relationship between broth supplement and high mutation yield has been examined in a series of "medial switch" experiments. This basic technique was exploited by Witkin (1956) in her now classical studies, and involves the incubation of treated cells on one medium for a given time with final selection on another. The difference between the two media lies in their relative degress of supplementation. The first medium may be either liquid or solid, whilst the second is usually the final plating medium. The experimental results are then usually expressed on a graph of mutation yield against the time of medial change,

although it must be recognised that reactions modifying mutant yield commenced on the first medium may continue upon the final plating medium (for discussion - see Kimball 1966).

In several experiments, EMS-treated cells of strain UV2 of B. subtilis were spread on filter membranes on the surface of selective agar plates containing trace supplements either of broth or of histidine and leucine, (respectively 1·5% v/v or 1·5 µg/ml of each). After two hours incubation at 37°C, the membranes were either left undisturbed, or transferred to a second agar plate of the same or different supplementation. Incubation was then continued until scoring. Results of such experiments are given in Table 17. Experiments 1-3 demonstrate clearly the higher mutation yield recovered on broth-supplemented agar. The two-hour period chosen for the first incubation was also sufficient for the broth to stabilise the great majority of mutants against the obviously deleterious effects of a broth-free medium.

In all experiments in Table 17, it will be seen that denial of broth during the first two hours, with or without broth in the subsequent incubation medium, led to an irreversible loss of mutants (MFD). This MFD was obviously independent of the concentration of specific requirements present, as experiments 4 and 5 demonstrate that these 4 substances in excess (30 µg/ml of each) in no way substituted for broth. A complete medium (NBA) proved as effective as broth-supplemented minimal agar when used as first medium in such experiments (results not shown).

TABLE 17. Mutation Fixation and MFD on Solid Media

MEDIUM I* Selective Agar plus:	MEDIUM II* Selective Agar plus:	MUTANTS PER MEAN OF THREE MEMBRANES IN EXPERIMENT NUMBER:				
		1.	2.	3.	4.	5.
Broth	Broth	275	82	467	-	-
Broth	Traces his, leu	251	98	453	-	-
Traces his, leu	Broth	53	36	131	57	85
Traces his, leu	Traces his, leu	55	23	132	50	66
Excess requirements	Broth	-	-	-	60	66
Excess requirements	Traces his, leu	-	-	-	32	61
BROTH THROUGHOUT		289	90	512	181	231
TRACES HIS, LEU THROUGHOUT		30	26	133	46	58

* Cells on filter membranes were changed from medium I to medium II after 120 minutes at 37°C with incubation continued to 4 days. Broth = 1·5% v/v NB. Traces his, leu = 1·5 µg/ml each of histidine and leucine, Excess requirements = 30 µg/ml each of histidine, leucine, uracil, and tryptophan.

MFD was also evident when cells were held on filter membranes on basal minimal agar plus glucose, with resuspension and plating at intervals (Figure 2). This MFD was rapid, only 20% of mutants arising on broth-supplemented agar on immediate plating being so recoverable after 90 minutes posttreatment. A small but genuine decline also occurred in the number of mutants recovered on agar with traces of histidine and leucine, as shown in Figure 2. In this last type of experiment, survival showed no appreciable decrease, the population changes being restricted to the mutant fraction. Observance of MFD on filter membranes on the surfaces of agar plates from which excess moisture had been removed must be borne in mind when explanations are postulated for MFD. It is in consequence unlikely that MFD on such solid media involves a "washing-out" of residual mutagen or mutagenic intermediates from treated cells.

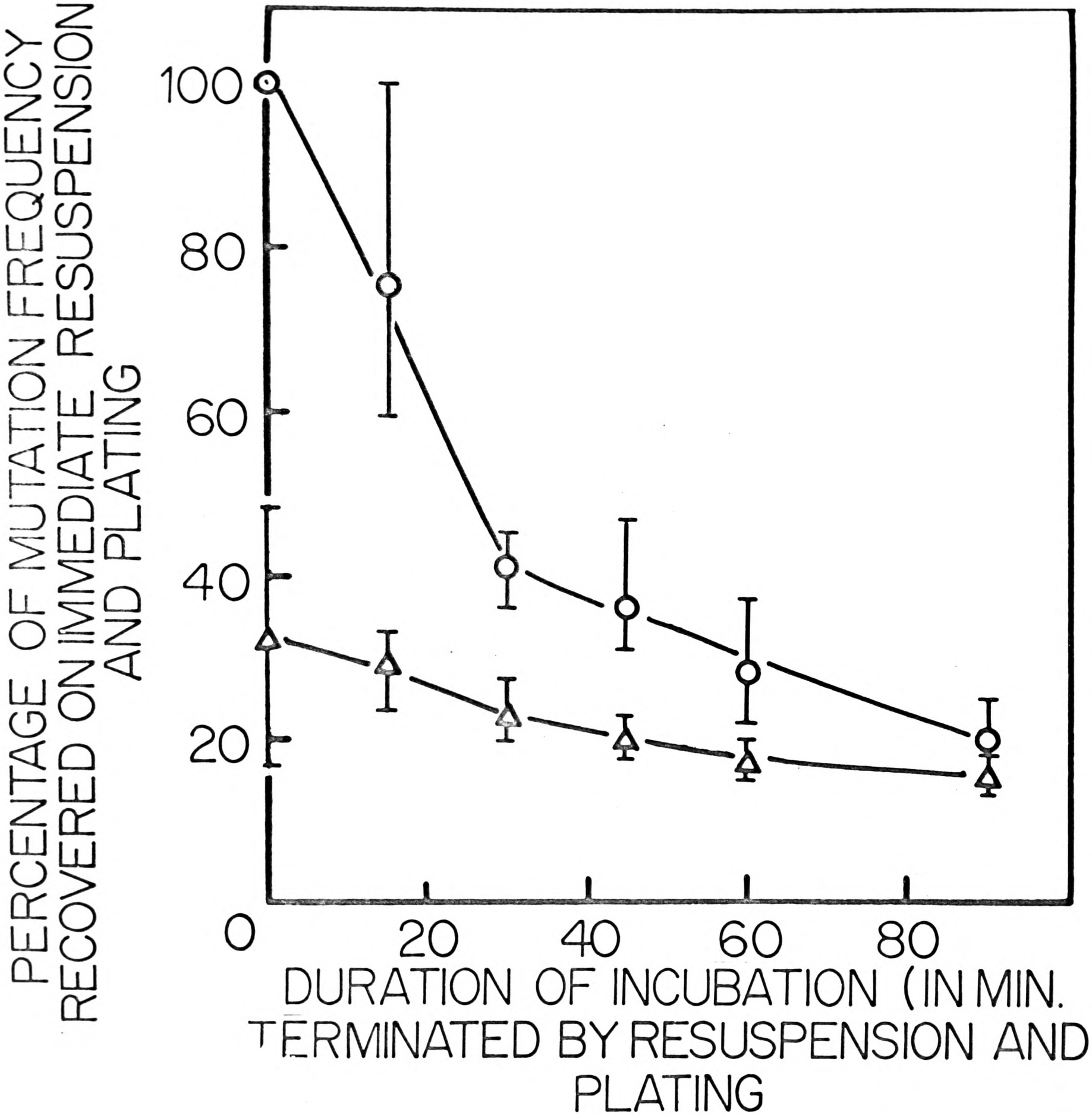
d. MFD in Liquid Media.

This discovery of a medial-dependent MFD on solid media was followed by further experiments of the medial-switch variety, in which EMS-treated cells were posttreated in liquid media before final selective plating.

The results of many such experiments are combined in Figure 3. It will be seen that a dramatic MFD took place in a minimal salts plus glucose medium, as indeed it had upon the agar-solidified equivalent. Whilst overall survival of treated cultures showed no decrease at all during these periods of posttreatment, only about 11% of the mutants recoverable on immediate plating on broth-supplemented agar

FIGURE 2.

MFD during incubation of populations of EMS-treated cells on filter membranes on minimal salts + glucose agar at 37°C with resuspension and plating at intervals for assay of mutant and total viable counts. Open circles = mutant yield on selective agar + 1·5% v/v nutrient broth, open triangles = mutant yield on selective agar + 1·5 μg/ml each of histidine and leucine. Each point is derived from the mean of at least 3 experiments, the horizontal bars being drawn between the extremes of experimental means.


PERCENTAGE OF MUTATION FREQUENCY RECOVERED ON IMMEDIATE RESUSPENSION AND PLATING
100
80
60
40
20
0
20
40
60
80
DURATION OF INCUBATION (IN MIN. TERMINATED BY RESUSPENSION AND PLATING

could still be obtained after 90 minutes posttreatment, and similarly about 30% of those recoverable on agar with traces of histidine and leucine. MFD is thus a phenomenon common to both liquid and solid media without broth.

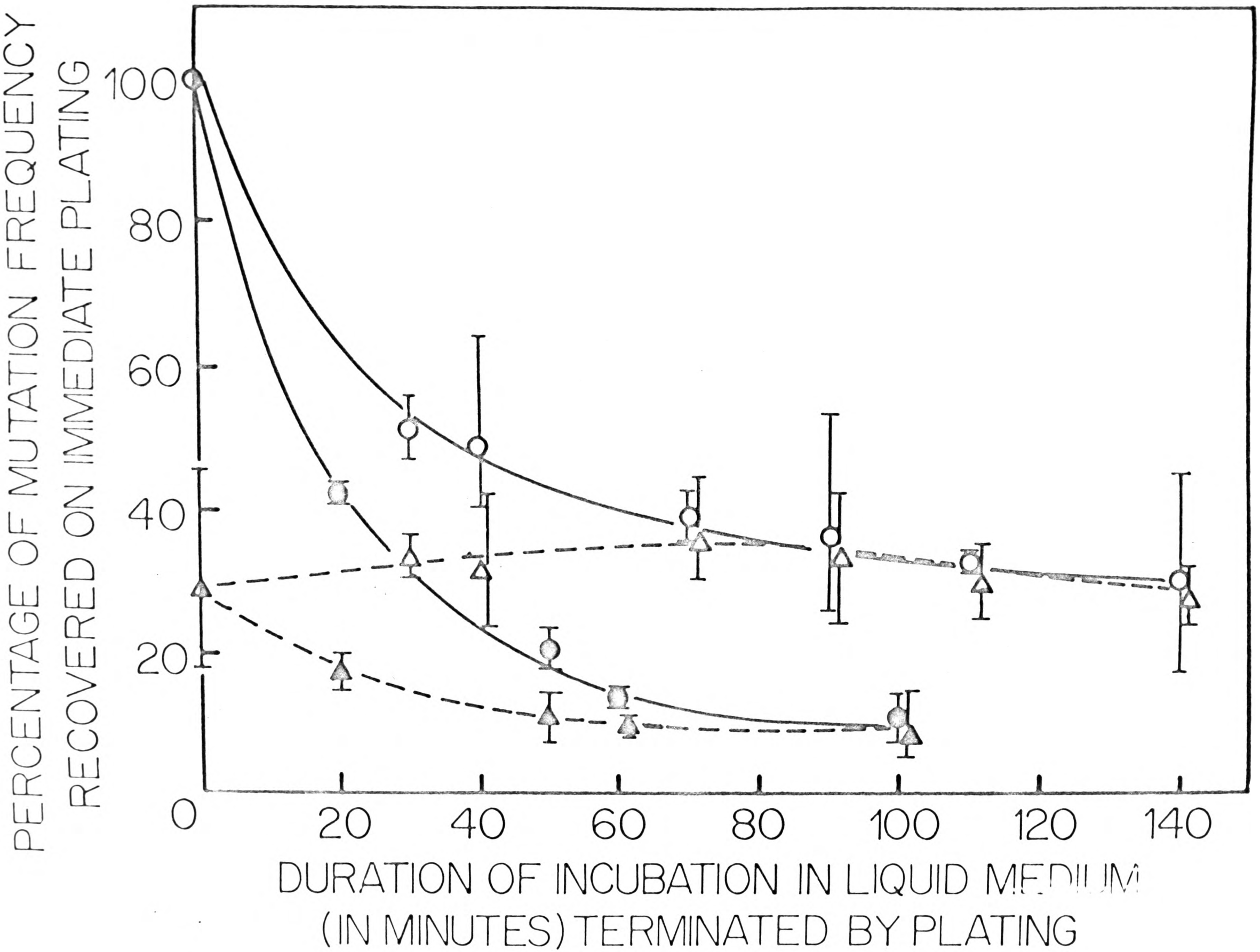
It has been shown in the preceding section that addition to selective agar of 1·5% v/v NB gave a high mutant yield even if after two hours on broth the membranes were transplanted to a broth-free medium. In most unexpected fashion, however, the comparable experiments with liquid media showed that broth produced no such effect, regardless of the final plating medium. The MFD curves in the liquid equivalents of the two plating agars (with or without broth or traces of histidine and leucine) follow very closely, although always slightly above, that of MFD in minimal salts and glucose liquid medium (shown in Fig. 3).

A second discrepancy between effects of solid or liquid medial posttreatment was shown on addition of the four growth requirements. In the preceding section, it was shown that their addition to solid media did not prevent MFD. Their addition to liquid minimal salts and glucose medium (at 30 μg/ml each of leucine, histidine, uracil, and tryptophan) reduced both the rate and extent of MFD of mutants recoverable on broth-supplemented agar. MFD of mutants recoverable on histidine and leucine-supplemented agar was completely abolished. The results of many such experiments are combined in Fig. 3.

Examination has shown that MFD was also spared on replacement of the four growth requirements by a mixture of

FIGURE 3.

MFD during incubation of EMS-treated cells in liquid minimal salts + glucose medium without enrichment (filled triangles and circles) or with added leucine, uracil, histidine, and tryptophan, each at 30 μg/ml. (Open triangles and circles.), with plating at intervals for assay of mutant and total viable cell counts. Circles = mutation yields on selective agar + 1·5% v/v nutrient broth, triangles = mutation yields on selective agar + 1·5 μg/ml. each of histidine and leucine. Each point is derived from the mean of at least 3 experiments. Horizontal bars are drawn between the extremes of experimental means.


PERCENTAGE OF MUTATION FREQUENCY
RECOVERED ON IMMEDIATE PLATING
100
80
60
40
20
0
20
40
60
80
100
120
140
DURATION OF INCUBATION IN LIQUID MEDIUM
(IN MINUTES) TERMINATED BY PLATING

casein hydrolysate, uracil, and tryptophan, or addition to them of 1·5% v/v NB. It has been revealed by analysis that the presence of only two of these growth requirements, leucine and uracil, was sufficient to produce the effect. This "leucine-uracil" sparing effect is most unlikely to be due to the metabolic combination of an exogenous supply of these two requirements and an endogenous supply of the other two, histidine and tryptophan: starvation of cells for 90 minutes of histidine and tryptophan before mutagen treatment in no way reduced the leucine-uracil effect.

These two discrepant phenomena, the absence of a broth effect in liquid medium, and the leucine-uracil effect, were then subjected to further experiment.

e. The Broth Effect Discrepancy.

A concentration of 1·5% v/v NB in otherwise selective medium permits MFD in liquid and prevents it on solid agar. A reason for this discrepancy may be sought at any level, and a number of lines of investigation were pursued. Before their results are described, a possible confusion of the reader may be avoided by the statement that the cause of this discrepancy has been found. It lies in the different relationship between cell concentration and the quantity of broth in the 2 sets of conditions. Its discovery will be described at the end of this section.

It is evident that many differences will exist between the liquid and solid states of the media described here, and in the responses of cells to them. A number of investi-

gations have been performed to elucidate these differences. The aim in each case has been to prevent MFD in liquid medium, with the premise that in so doing its mechanism might be partially uncovered.

1. The Omission of Medial Components: compared to liquid minimal medium with glucose, the omission of one or more components from the medium gave only a slight sparing of MFD. Omission of glucose, ammonium sulphate, and/or sodium citrate, or the use of such simple suspending liquids as 0·85% w/v saline or phosphate-buffered saline, proved in no way as MFD-sparing as the effect of leucine and uracil just described. In addition, some reduction in overall viability took place in most omission media. Although in no way comparable to leucine and uracil, the most effective omission medium in this regard was liquid minimal salts without both citrate and glucose. This suggests that the processes responsible for MFD in a liquid medium, whilst not requiring addition of an energy source under the tested conditions, acted at maximal efficiency in the presence of one.

These experiments do however demonstrate that MFD cannot be inhibited by the omission of a medial component. This is in striking contrast to findings with the lactose fermentation mutation system in E. coli B (Doudney and Haas 1958). In their experiments, MFD did not occur in a minimal medium after UV, although it was quickly detected on omission of one or more components of the medium, or in saline. It is of possible relevance that an enhancement in the presence of an energy source has also been shown for the breakdown of

DNA in B. subtilis following treatment with another alkylating agent, MMS, or UV (Searashi and Strauss 1967).

2. Osmotic Stabilisation of the Medium: it is known from the experiments of Strauss (1961) that so-called permeability defects arose in E. coli strains treated with another ethylating agent, diethyl sulphate. (These defects deserve further investigation following the more recent discovery of excision (and in some cases repair) of alkylated lesions in DNA, as they were simply detected as the release of nucleotide-containing material into the medium.). Known effects of alkylating agents are of course by no means incompatible with damage to other cellular components. It seemed possible that a progressive increase in osmotic instability of a fraction of the treated cell population, including the majority of mutants, might occur in liquid medium with or without broth. Instability might then lead to the loss of mutants, either in the liquid medium or during the additional stresses of plating (Hardigree and Adler 1967, Markovitz and Baker 1967).

Two facts speak against this explanation however. Firstly, osmotic stabilisation of the posttreatment liquid medium with either 0·5 M saline or 20% w/v sucrose produced no MFD-sparing effect. Secondly, as stated in MATERIALS AND METHODS section 5, substitution of in-plating for spreading of cell inocula gave approximately the same result in terms of mutant recovery. As thoroughly as is possible, therefore, osmotic instability has been ruled out as a cause of MFD in liquid media.

3. The Possible Contribution of an Agar Component: the occurrence of a broth effect only upon solid medium may indicate that it is produced by an agar component in company with added broth. Absence of this postulated agar component would lead to MFD in liquid media regardless of their degree of supplementation. Attempts were therefore made to extract such a component from agar. Concentrated agar gels (more than 10% w/v) were exposed to liquid medium (with or without broth) for many days in the cold, and the liquid then filtered off and used as suspending fluid for mutagen-treated cells. Although it was obvious from the pale straw colour of the liquid medium that substances were eluted from the agar gels, MFD proceeded normally in these liquids regardless of the presence of broth. A complementary experiment was performed on solid medium with the substitution of a purer agar (Oxoid Immunodiffusion Agar) for the agar normally used (Davis New Zealand Agar). Mutation frequency showed the same medial dependence with this purer agar, and MFD proceeded in normal fashion. This evidence makes it highly unlikely that the broth effect involved the participation of an agar component.

4. Random Tests for Possible MFD Inhibitors: caffeine at 0·5 mg/ml or a mixture of non-specific amino acids (30 μg/ml each of glutamic and aspartic acids and proline proved powerless to stop MFD in broth-supplemented liquid medium.

5. Residual Mutagen or Mutagenic Intermediates: it is evident that the presence in treated cells of residual mutagen or its mutagenic intermediates after the termination of treat-

ment may provide a source of complication in studies of chemical mutagenesis. This does not refer to delayed effects of mutagen-DNA interaction per se, such as depurination or the posttreatment continuation of phage inactivation (Lawley and Brookes 1963, Brookes and Lawley 1964, Loveless 1966), but to the maintenance of unreacted mutagen in some form within cells after the cessation of direct mutagen treatment. This explanation has been tendered by Kølmark and Kilbey (1962) to explain an "after-effect" in Neurospora crassa. After termination of direct treatment with DEB, induced mutation frequency increased if conidia were stored at 22-30°C in the form of a centrifuged pellet. Plating or vigorous shaking of the conidia in water at 22-30°C rapidly eliminated the after-effect. A similar phenomenon has more recently been described for the monomer of DEB, EO (Kilbey and Kølmark 1968).

Such residual mutagen, or mutagenic intermediates, immune from the thiosulphate-washing procedures, might be responsible for the phenomenon described in B. subtilis. The implication would then be that the production of high mutation yields on broth-supplemented agars was due to a combination of direct mutagen action with effects of residual mutagen. These effects must take place within two hours, although shaking in liquid medium of any composition, or plating on a broth-free medium, would remove the contribution of residual mutagen. This hypothesis fits quite well with the data presented here, but it may be excluded for a number of reasons. Firstly, it is difficult to explain

how leucine and uracil could reduce washing-out of residual mutagen in a specific manner. Secondly, EMS-induced mutation frequency in nonlysogenic strains of E. coli K12 was not changed by posttreatment agitation in minimal medium or buffer (Schwartz 1963, Verly, Barbason, Dusart, and Petispas-Dewandre 1967). There is therefore no evidence in an admittedly different species of a retention of mutagen. Finally and conclusively, it has been subsequently shown that MFD could be abolished by adjustment of the cell concentration relative to that of broth.

Attempts were made nevertheless for comparative purposes to demonstrate an after-effect, perhaps due to residual mutagen. By analogy with the DEB-Neurospora experiments, after thiosulphate termination of EMS treatment cells were held as pellets in centrifuge tubes. After 2 hours storage, the cells were resuspended and plated for mutant recovery and overall survival. Results of two such experiments are given in Table 18. It will be seen that no increase in mutation frequency occurred such as might be attributable to residual mutagen. Two points are worthy of comment. Firstly, lethality in pellets held at 37'C increased in a manner similar to that of untreated control cells, thereby demonstrating the autolysis to which the transforming strains of B. subtilis are particularly prone. Secondly, no MFD was evident in pellets stored either at 0 or 37'C, presumably due to metabolic inactivity imposed by the mode of storage.

6. Temperature Relationships: the temperature relationships

TABLE 18. The Effect of Storage of EMS-treated Cells as Centrifuged Pellets on Mutagen Frequency and Viability of strain UV2. - 2 experiments.

POSTTREATMENT	EXPERIMENT NUMBER	Viable Cells per ml x 10^8		Mutants scored per ml.		Mutants per 10^7 survivors	
		* SAB	SAhl	SAB	SAhl	SAB	SAhl
IMMEDIATE RESUSPENSION AND PLATING	1	1·93	1·98	4390	1010	228	52
	2	1·20	1·29	1610	480	134	37
HELD AS A PELLET FOR TWO HOURS AT 0'C, RESUSPENDED AND PLATED	1	1·66	1·47	3570	990	215	66
	2	1·23	0·92	1590	520	129	57
HELD AS A PELLET FOR TWO HOURS AT 37'C, RESUSPENDED AND PLATED	1	0·92	0·74	2120	580	231	78
	2	0·46	0·42	670	220	146	52

* SAB = selective agar plus 1·5% v/v NB. SAhl = selective agar plus 1·5 µg/ml each of histidine and leucine. Each figure is derived from the mean of three plate counts.

of MFD have been further examined by repeating liquid post-treatments at low temperatures. While MFD was reduced in rate at 22'C compared to 37'C, posttreatment at 0-1'C totally abolished it, regardless of the supplementation of the medium (Figure 4.). Increasing the temperature of incubation to 37'C after as much as 80 minutes at 0-1'C initiated the MFD process at normal rates (Figure 5.). These temperature-effect experiments do not unfortunately contribute much to our knowledge of MFD, although they do stress its probable dependence on cellular metabolism.

7. Liquid Holding Recovery: the decline of EMS-induced mutation frequency in B. subtilis is analogous in its dependence on temperature and independence of medial composition to the Liquid Holding Recovery (LHR) described by Roberts and Aldous (1949) for the restoration of viability of UV-irradiated cells of E. coli strain B. This LHR took place in medium of any composition tested, from the nutritionally rich NB to distilled water. Repair mechanisms have been implicated in the reduction of lethality of E. coli strains in liquid media (Castellani, Jagger, and Setlow 1964, Harm 1966), perhaps during a division delay induced by the liquid treatment (Jagger, Wise, and Stafford 1964). A corresponding phenomenon of reduction of UV-induced mutation has been described by Zetterberg (1966) with the fungus Ophiostoma multiannulatum. Fewer mutants were recovered from irradiated conidia of this organism after posttreatments in liquid medium. The discrepant behaviour toward broth demonstrated by B. subtilis could well be explained by such a

FIGURE 4.

Effect of temperature on EMS-MFD in liquid media. Inhibition of MFD at 0-1°C in liquid minimal salts + glucose medium without other enrichment (left hand graph - <u>filled</u> symbols), or with added casein hydrolysate (to 0·02% w/v) and uracil + tryptophan (30 μg/ml each) - (right hand graph - <u>open</u> symbols), with plating at intervals for assay of mutant and total viable counts. <u>Circles</u> = mutation yields on selective agar + 1·5% v/v nutrient broth, <u>triangles</u> = mutation yields on selective agar + 1·5 μg/ml each of histidine and leucine. Each point is derived from the mean of at least three experiments. Horizontal bars are drawn between the extremes of experimental means.

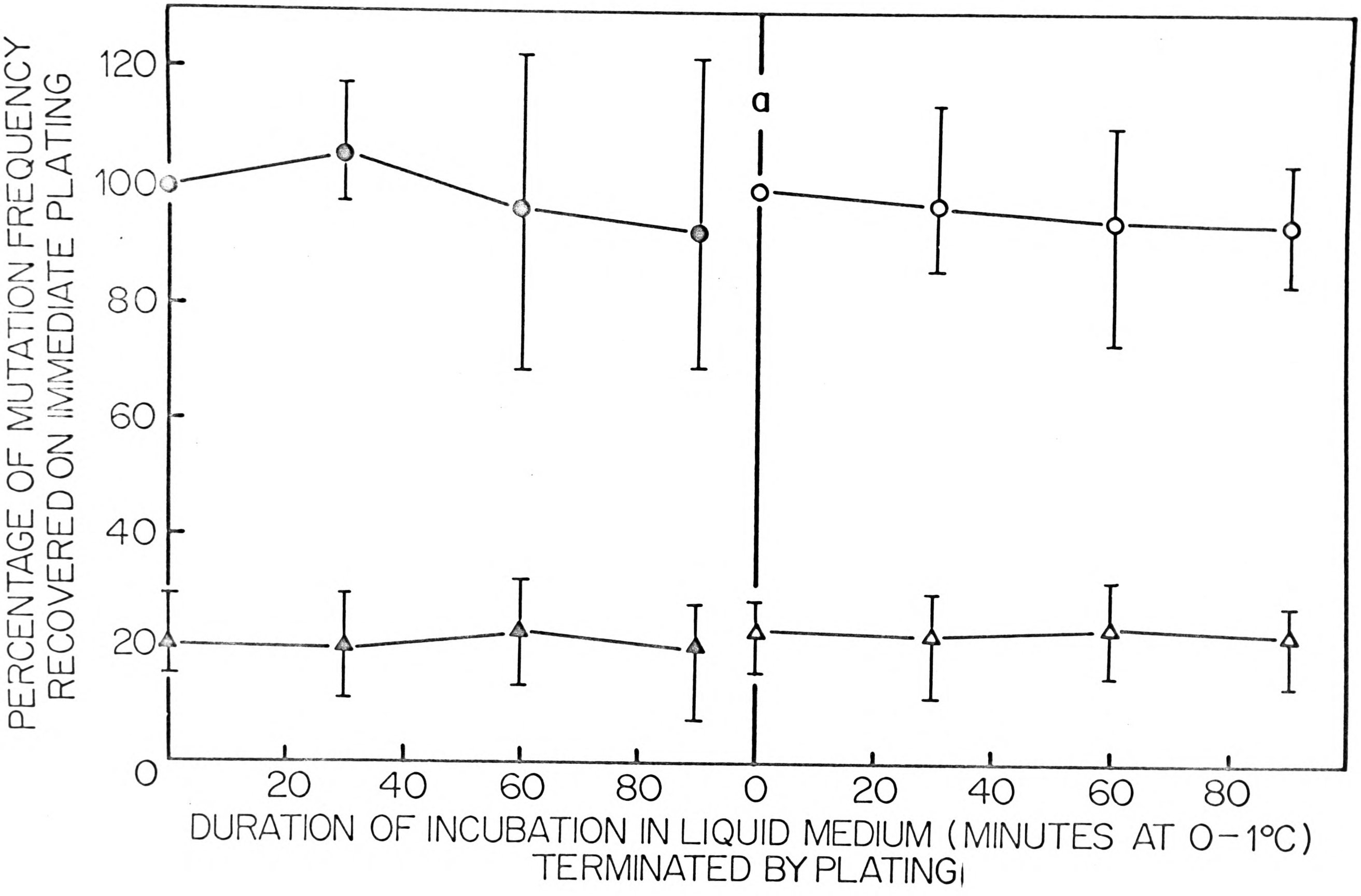

PERCENTAGE OF MUTATION FREQUENCY RECOVERED ON IMMEDIATE PLATING
120
100
80
60
40
20
0
a
20
40
60
80
0
20
40
60
80
DURATION OF INCUBATION IN LIQUID MEDIUM (MINUTES AT 0–1°C) TERMINATED BY PLATING

FIGURE 5.

Commencement of MFD after inhibition at 0-1'C on raising the temperature of incubation to 37'C. Plating medium in all cases is selective agar + 1·5% v/v nutrient broth, and the incubation liquid = minimal salts + glucose medium at 0-1'C (open circles) or at 37'C (filled circles). Each point is derived from the mean of at least three experiments. Horizontal bars are drawn between the extremes of experimental means.

b

PERCENTAGE OF MUTATION FREQUENCY RECOVERED ON IMMEDIATE PLATING

100
80
60
40
20
0

20 40 60 80 100 120 140 160

DURATION OF INCUBATION PERIODS IN LIQUID MEDIA (IN MINUTES)

recovery mechanism specific to liquid medium, although results presented in the next section invalidate this hypothesis at birth. LHR in E. coli has been studied by genotypic and phenotypic interference with the repair mechanisms presumably involved (Harm 1966), as has MFD (Witkin 1966). It will no doubt be instructive in the future to study MFD in B. subtilis in the same way.*

8. Relationship between Concentrations of Cells and Broth: it is instructive to compare the amounts of broth available to cells under the different conditions of solid and liquid incubation. In liquid medium, cells were posttreated at a density of 1-3 x 10^9 per ml in a medium containing 1·5% v/v NB. On solid medium on the other hand, inocula of 1-3 x 10^7 cells were routinely spread on 25-30 ml of agar with the same 1·5% NB. There is thus a difference of more than 1000-fold in the broth:cell ratio in the two conditions. It is possible that the lack of broth effect found in liquid media results simply from an insufficiency of broth.

If this was true, it was argued, it should be possible to reduce or abolish MFD in liquid medium by increasing the broth concentration. Three replicate experiments were then performed in which EMS-treated cells were posttreated in a liquid medium with 50% v/v NB, a 33-fold increase in broth concentration. The result of one of these experiments is shown in Figure 6, the others giving essentially similar results. It is quite clear that the MFD-sparing effect of

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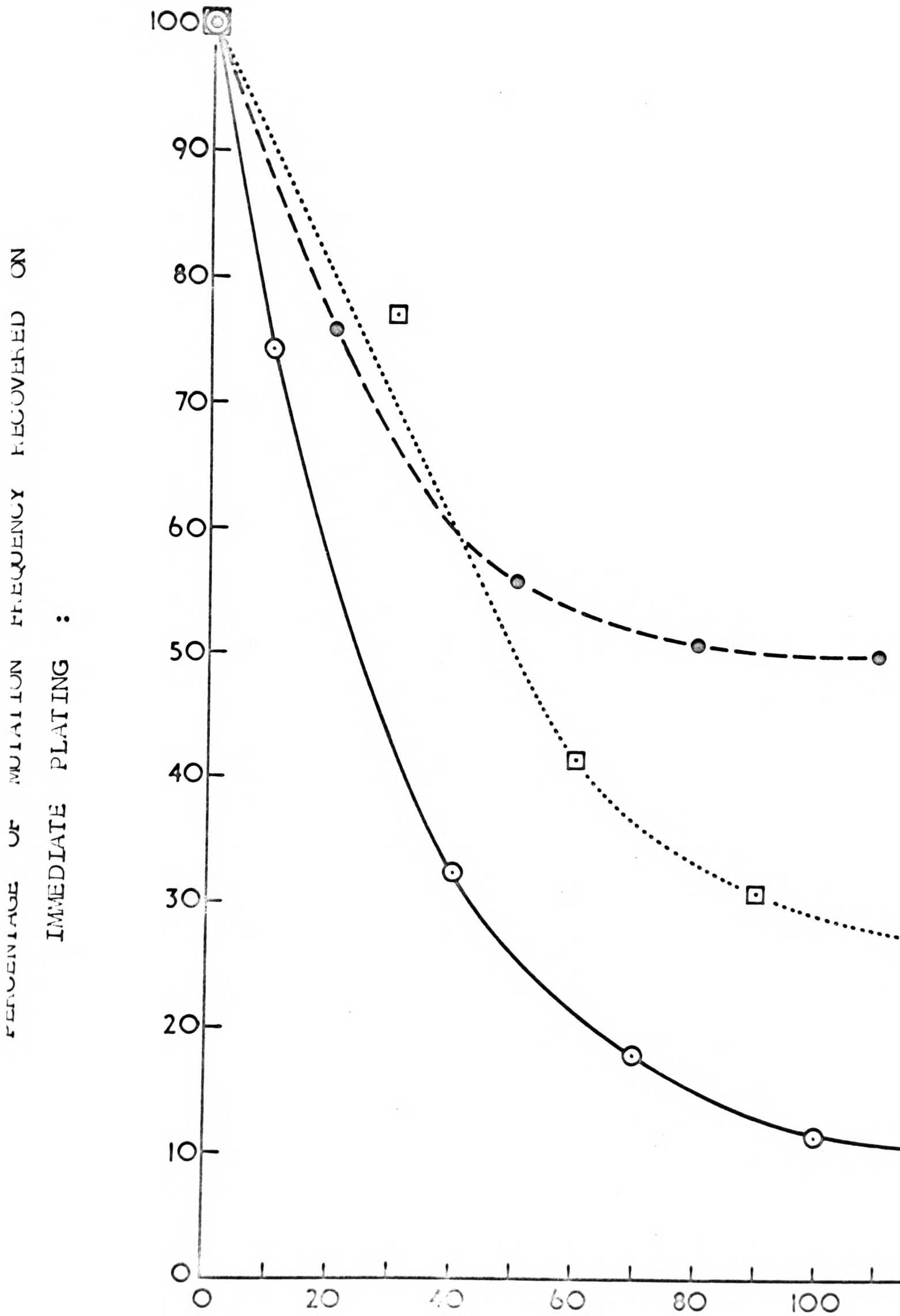
* This work has been published as Corran J. Molecular and General Genetics. 1968. 103. 42-57.

FIGURE 6.

A comparison of MFD in various liquid post-treatment media : a single representative experiment. Plating medium = selective agar + 1·5% v/v nutrient broth. Filled circles and broken line = incubated in minimal salts medium + glucose + 30 μg/ml each of histidine, uracil, tryptophan, and leucine. Open squares and stippled line = incubated in 50% minimal salts medium + glucose + 50% v/v nutrient broth. Open circles and entire line = incubated in minimal salts + glucose medium without further enrichment.


PERCENTAGE OF MUTATION FREQUENCY RECOVERED ON IMMEDIATE PLATING :
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
DURATION OF INCUBATION IN LIQUID MEDIUM (IN MINUTES) TERMINATED BY PLATING :

50% v/v NB in no way approached that of 30 μg/ml each of leucine and uracil. Although some reduction of both rate and extent of MFD was evident, the experimental results do not suggest that the lack of broth effect in liquid medium was due to broth shortage. The small amount of sparing detected could indeed be due to the leucine and uracil content of the 50% v/v NB. Parallel experiments showed that 100% v/v NB showed little, if any, more sparing of MFD.

This negative finding and conclusion, reached early in this work, led in fact to the numerous investigations of trivia just reported. A conversation with Dr. E.M. Witkin (in autumn 1968) stimulated new investigation of the problem. Dr. Witkin reaffirmed in her own experiments (subsequently published as Witkin 1968) that the dilution at which cells (treated with UV in her case) were plated or posttreated was critically important for the recovery of high mutant yields.

A number of experiments were then performed, reciprocal to those which had already given these negative results. Instead of raising the concentration of broth while maintaining a high cell density, the cell population was sequentially diluted in a constant low concentration of broth. The results of these experiments are summarised in Tables 19 and 20.

Table 19 illustrates the posttreatment dependence of the inhibition of MFD in liquid medium by 1·5% v/v NB on the cell concentration. At a dilution factor of 10^{-2}, corresponding to 1-3 x 10^7 cells per ml depending on the experiment, with the cells concentrated before plating, MFD was totally prevented. It should be noted that the apparent

TABLE 19. The Dependence on Cell Dilution of MFD and Broth Effect in 1·5% v/v NB-supplemented Liquid Minimal Medium.

TIME OF LIQUID POSTTREATMENT IN MINUTES	% INITIAL MUTATION FREQUENCY RETAINED AT A DILUTION OF:		
	10^0	10^{-1}	10^{-2}
0	100	100	100
40	39·4	80·3	105·3
80	28·2	55·1	90·5*
120	23·2	54·5	60·9*

* reduction in mutation frequency not due to a decrease in mutant number, but to an increase in the viable count. No other change in viable count was observed. Each figure represents the mean of at least three experiments. Dilution 10^0 was equivalent to cell concentration of 1-3 x 10^9 per ml. Plating was on broth-supplemented selective agar.

MFD at 120 minutes of posttreatment at this 10^{-2} dilution does not in fact represent a decrease in mutant number, but an increase in viable count. This presumably reflects the recommencement of cell division of the nonmutant cells. MFD was much reduced even at the lower dilution of 10^{-1}, when the stabilisation of mutant yield at about 50% of the initial value implies that the broth:cell ratio is of cardinal importance to the prevention of MFD immediately after post-treatment begins.

Table 20 records the relevant control experiments in liquid minimal salts plus glucose, and it will be seen that maximal MFD in this medium was independent of cell concentration. MFD at various cell concentrations was also similar in liquid minimal salts medium plus 30 μg/ml each of uracil and tryptophan, with or without 1·5 μg/ml each of histidine and leucine.

The successful prevention of MFD by dilution and the absence of this effect in the reciprocal experiments pose another question. High cell densities with increased amounts of broth, it will be remembered, showed only a small sparing of MFD (Fig. 6). This may still be attributable to the broth:cell ratio, which is yet about 30-fold less than that necessary to prevent MFD totally in liquid medium. An alternative explanation, however, might be that the previous growth history of cells with respect to broth governs the extent of their subsequent response to broth in post-treatments. NB and a minimal medium with only required

TABLE 20. The Lack of Dependence on Cell Dilution of MFD in Liquid Minimal Salts plus Glucose Medium in the Absence of Broth.

TIME OF LIQUID POST-TREATMENT IN MINUTES	% INITIAL MUTATION FREQUENCY RETAINED AT A DILUTION OF: 10^{0}	10^{-1}	10^{-2}
0	100	100	100
40	20·1	27·6	26·2
80	10·7	12·9	13·6
120	11·6	11·3	10·5

Each figure represents the mean of at least three experiments. Dilution 10^{0} is equivalent to cell concentrations of 1-3 x 10^{9} per ml. Plating was on trace-broth supplemented agar. No change in viable count was detected in any sample.

supplements are indeed two very different cultural regimes, and it is quite possible that growth in one medium renders cells incapable of taking advantage soon enough of the other in posttreatment experiments. It is hoped to explore this possibility in the future.

It was of interest to determine the relative sizes of broth effect shown by cells grown up in different media. The broth effect is defined for the purposes of this investigation as the increase in mutation frequency from aliquots of the same treated suspension upon selective agar supplemented with traces of histidine and leucine, (or other specific requirements dependent on the marker(s) studied). Experiments have shown that the yield of mutants on broth-supplemented agar does not vary greatly at particular doses dependent on the previous growth medium of the cells. In contrast, mutant yield on agar supplemented with traces of histidine and leucine decreased as the complexity of the previous growth medium increased. With its upper limit relatively constant therefore, and a reduction in its lower limit, the broth effect increased with increasing complexity of the preparative medium for cells. The greatest broth effect was obtained from cells prepared in 100% v/v NB, followed in descending order by 50% v/v NB in minimal medium, specifically supplemented minimal medium plus 1·5% v/v NB, and specifically supplemented minimal medium alone. It is really not so surprising that the richer the medium used to prepare a cell population, the fewer are its revertants on the more challenging selective agars.

SUMMARY OF THE BROTH EFFECT: a broth effect has been found for the EMS-induction of $su^{+}_{his\text{-}Al\ leu}$ mutants in B. subtilis. This is demonstrated as a 3-4-fold increase in the frequencies of mutation recovered on media containing traces of broth compared to that on media containing traces of the previous growth requirements. The effects of broth can be mimicked by casein hydrolysate (with the tryptophan in which it is deficient, and uracil), thus demonstrating that the amino acid content of the broth is the stimulatory agent. Amino acids other than histidine and leucine are involved as a better supply of these two amino acids does not raise mutation yield in the manner of broth. Denial of broth in liquid or on solid media leads to MFD, detectable as reduced mutant yield on subsequent plating on broth-supplemented agar. The presence of broth (1.5% v/v) prevents this MFD on agar, but not in liquid media unless the population density is lowered by dilution. This signifies the importance of the ratio of cell number:broth concentration in the prevention of MFD. No other specific means have been found to prevent MFD, although it can be delayed at 0-1°C. There is no evidence for the participation in the broth-related phenomena described of mutagen-induced osmotic instability, involvement of an agar component, residual mutagen, or liquid holding recovery.

f. The Leucine-Uracil Effect Discrepancy.

It has been shown that the addition of the four growth requirements of the strain during posttreatment spared MFD in liquid medium but not on solid medium. It was subsequently

found that only two of the specific requirements were necessary to produce the effect in liquid, hence the designation "leucine-uracil effect". These two substances produced their effect even when cells had been starved of the other two requirements before mutagen treatment. As this starvation period was of a duration approximately equal to one cell doubling in the media used, it appeared that the MFD-sparing produced by leucine and uracil did not require active synthesis.

Unlike the broth effect, but in similar fashion to MFD, the liquid-specific leucine-uracil effect was relatively independent of cell density at a given concentration of the two substances. This is illustrated in Table 21. If anything, the effect was slightly reduced at the lowest cell concentration used, 10^{-2}, equivalent to 1-3 x 10^{7} cells per ml, dependent on the experiment. It is most probable that the phenomenon is caused by the interference of leucine and uracil with the processes involved in MFD, for some unknown reason in a liquid medium only, although a general effect on the strain cannot be excluded with respect to other properties. Leucine, for example, is already known to inhibit certain Aspergillus mutants (Pontecorvo 1964), to kill a strain of E. coli K12 in the absence of its required amino acid, phenylalanine (Kindler and Ben-Gurion 1965), and to affect the ability of a strain of E. coli K10 to take up amino acids from the medium (Inui and Akedo 1965).

The leucine-uracil effect would be of greatest interest to the investigator if a specificity of action could be

TABLE 21. The Lack of Dependence on Cell Concentration of the Leucine-Uracil Effect on MFD in Liquid Minimal Salts plus Glucose with 30 μg/ml each of leucine and uracil, but without broth.

TIME OF LIQUID POST-TREATMENT IN MINUTES:	% INITIAL MUTATION FREQUENCY RETAINED AT A DILUTION OF:		
	10^{0}	10^{-1}	10^{-2}
0	100	100	100
40	51·3	52·8	45·9
80	44·9	43·9	33·5
120	34·8	38·7	25·9

Each figure represents the mean of at least three experiments. Dilution 10^{0} was equivalent to cell concentrations of 1-3 x 10^{9} per ml. These experiments were performed simultaneously with those recorded in Tables 19 and 20. The control for these experiments in liquid minimal medium plus glucose only is given in Table 20.

described. This point was examined by studying MFD of other markers after the induction of their reversion with EMS. The markers studied were three of those detailed in section 5d of RESULTS as highly revertible with EMS, namely ser-gly (EMS78), tyr (EMS 110), and threo (AP3). All three markers when tested as monoauxotrophs showed MFD after reversion induction with EMS. None however showed a leucine-uracil effect, the implication thus being that the effect is a characteristic of strain UV2.

Each marker was then separately introduced by transformation into strain UV2. MFD both of revertants of the new marker and of supersuppressor revertants of the his-Al and leu markers were then studied in the same experiments, also the leucine-uracil effect on each type of mutant. It is stressed that supersuppression of each of the three new markers by $su^{+}_{his\text{-}Al\ leu}$ has already been excluded (RESULTS section 5d): this study is genuinely of two mutant types, not of the same mutation on different plating media.

In none of the three instances was the leucine-uracil effect specific for the su^{+} mutation. Mutants to independence of serine + glycine, tyrosine, and threonine in their turn showed approximately the same amount of leucine and uracil-induced sparing of MFD as the su^{+} mutants simultaneously induced. The leucine-uracil effect is thus a general property of strain UV2. It is of interest that leucine and uracil alone were insufficient to produce the full sparing effect on the two types of mutants in each new substrain; although some sparing was noted, its extent differed in the

different substrains. In each case, addition of the extra requirement imposed by the new marker was necessary for sparing of MFD to the level originally described, and for both types of mutant.

It has not been possible to further elucidate the leucine-uracil effect. It is hoped that, in the future, investigation of the basic strain UV2 by sequential transformation of its four requirements to prototrophy will allow identification of any responsible allele. Alternatively, effects of physiological conditions will be examined.

The results presented here also show that the MFD property is not restricted to the su^+ mutation, but that it also occurs for reversion to prototrophy of three other markers which are not subject to the same suppressors. In view of the possible suppressor-specificity of such phenomena as the broth effect and MFD, it is essential that the nature of the revertants of these three other markers be elucidated. The necessary techniques are laborious, however, and have not yet given a definite result.

SUMMARY OF THE LEUCINE-URACIL EFFECT DISCREPANCY: MFD was considerably spared in liquid medium by two of the growth requirements of the strain, leucine and uracil. No such sparing occurred on solid medium. The participation of active synthesis in this effect seems unlikely as starvation of the cells for the other two growth requirements did not reduce the sparing. Unlike the broth effect, but like MFD, the effect of leucine and uracil was relatively independent

of cell density. The effect is not specific, producing approximately the same degree of MFD-sparing of revertants of other markers added to the strain as of supersuppressor revertants. These other markers were however unaffected by leucine and uracil sparing of MFD when tested as monoauxotrophs, where MFD took place "normally". This suggests that the effect is a characteristic of strain UV2, either a response to certain physiological conditions, or caused by one or more of the four mutations known to be carried by the strain. MFD has also been shown here to affect at least three markers in addition to the supersuppressor revertants, and to approximately the same extent.

q. Effects on MFD of Very Short Periods on Rich Medium.

Studies to date have demonstrated a great similarity in the medial dependence of induced mutation in EMS-treated B. subtilis and UV-treated E. coli. The sole difference so far discerned is the effect of leucine and uracil (or of more complex mixtures containing these two substances), though even this probably reflects a strain- rather than a species-specificity.

Some paradoxes still remain unexplained in the E. coli systems with which most of the work has been done. For example, it was shown by Witkin (1963) that exposure of UV-irradiated cells to nutrient agar for a very short time prevented subsequent MFD in liquid minimal medium but not on minimal agar. The MFD normally promoted by saline incubation or chloramphenicol challenge was not inhibited either,

and photoreactivability of mutation remained unaffected. The shortest exposure to nutrient agar physically manageable, 30 seconds, was sufficient to produce the effect. No hypothesis has yet been offered in explanation of this extraordinary happening, although the interference of nutrients with recovery processes is known, e.g. the blocking of LHR in E. coli B (and K12 rec^-) after UV by traces of yeast extract (Ganesan and Smith 1968). In the present case however, it is the shortness of the time required on rich medium to inhibit liquid MFD that defies explanation.

It was of interest to search for such paradoxical situations in B. subtilis. EMS-treated cells of strain UV2 were accordingly given a very short incubation on a very rich medium before liquid posttreatments were applied. After 2 minutes on the surface of filter membranes on BHIA plates, cells were removed from the membranes into liquid medium by vigorous shaking. The relatively "long" period of two minutes on BHIA was given to increase the chance of detection of any effect. The exposure to BHIA was at 37'C, and control samples were treated on BHIA at 0-1'C, and on minimal agar at both temperatures. Cells were then eluted from the membranes and studied for MFD in liquid media as before.

A very slight MFD-sparing effect was detected with BHIA-treated cells, whether the treatment had been at 0 or 37'C. No MFD-sparing was given by two minutes on minimal agar at either temperature. It was subsequently shown that this slight sparing of MFD after 2 minutes on BHIA could be totally

attributed to the nutrients picked up from the BHIA by the filter membrane and carried over into the liquid medium used for resuspension. Reconstruction experiments have shown that the sparing could be mimicked either by charging the membrane with traces of liquid BHI, or by the addition to cells held on minimal agar of a sterile membrane held on BHIA for 2 minutes. There was no effect of 2 minutes on BHIA on the leucine-uracil effect, or on MFD in broth-supplemented liquid minimal medium.

There is thus no evidence with EMS-treated B. subtilis of the inhibition of MFD in liquid by such pulse treatment on rich solid medium.

7. The Induction of Supersuppressor Mutants of B. subtilis with UV.

a. Comparison of the two available techniques.

It was apparent from reversion spot-tests that the his-A1 marker responded less to UV than to EMS (Table 4). It was then found that the overwhelming majority of induced his-A1 revertants were in fact supersuppressor revertants giving simultaneous independence of leucine (Table 13a,b). It was therefore of interest to study their induction by UV. For this mutagen, the treatment of large cell populations is indicated for two reasons. Firstly, UV is notably less mutagenic in the studied system than EMS. Therefore either higher, possibly more mutagenic, doses or larger populations must be studied. Secondly, the known ability of UV to

induce defective prophages carried by B. subtilis (Subbaiah et al. 1965) provides an additional complication. This problem can be avoided however if low UV doses are used, with little killing, either directly by UV or indirectly by phage induction. In consideration of these two factors, it is evident that both low doses and large populations are required for study.

Two techniques are available for the study of UV-mutagenesis. In the first technique, UV-treated cells may be directly spread on selective agars, with or without trace supplementation, i.e. as described for EMS. The same media as used with EMS may be used here, although cells must be plated at higher density (c. 10^9 per plate compared to c.10^7 per plate) due to the lower mutagenic efficiency in this system of UV. As will be seen, mutants were never recovered in large numbers. The second technique has been described by Jensen and Haas (1963), and modified here as the "filter membrane technique". Cells are irradiated on the surfaces of filter membranes and then incubated on membranes on NBA plates for two hours. Selection for induced mutants may then be made by simply lifting the filter membrane on to a selective medium and continuing incubation. Two technical points must be mentioned:

1. It has been proved that a two hour period on NBA is ample time for the fixation and expression of induced mutants. Experiments in which the NBA incubation time was varied showed that mutation yield reached a maximum after about 90 minutes on rich medium with little or no subsequent increase (see the

representative experiment in Figure 7).

2. In practice, it was found that nutrient carry-over from NBA to selective agar promoted residual division of the nonmutant survivors, resulting in diminished size of the mutant colonies. A 30-minute period of starvation was therefore interposed between NBA and selective agar, as in the following diagram:

STEP 1 ⟶	STEP 2 ⟶	STEP 3 ⟶	STEP 4 ⟶	STEP 5
Place cells on filter.	UV irradiate.	2 hours on NBA at 37°C.	30 mins. on minimal agar at 37°C.	selective medium up to 4 days at 37°C.

Careful study has shown that the starvation period of step 4 has no effect on mutant yield, the mutants obviously being rendered immune to medial changes by their two hours on NBA. Starvation up to $2\frac{1}{2}$ hours had no effect on mutant yield, although 30 minutes was adopted as satisfactory for the reduction of nutrient carry-over.

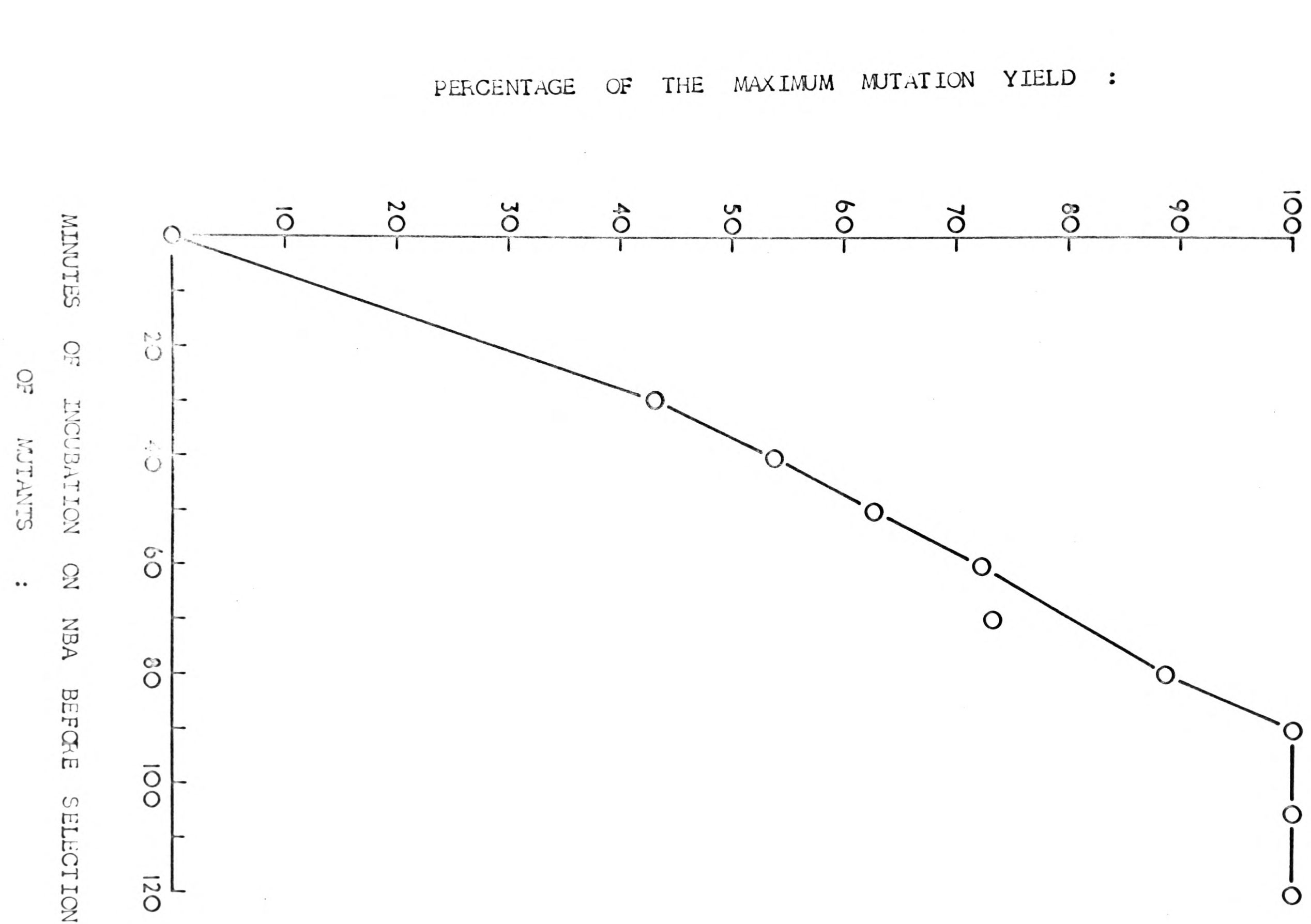
The advantage of this technique lies in the larger cell populations that may be loaded on to the filter membranes. Greater numbers of mutants may thus be recovered after the same low doses of UV. The disadvantage, however, is that all chances of comparison between plating media are lost. In section 8 of RESULTS, EMS mutagenesis is directly compared with UV-mutagenesis using this technique.

b. Results of the Direct Spreading Technique after UV.

The induction by UV of the supersuppressor mutants scoreable in this system has been confirmed in dose response

FIGURE 7.

The fixation and expression of UV-induced supersuppressor revertants as a function of increasing incubation time on Nutrient Broth Agar. Aliquots of cells were placed on filter membranes, given a uniform UV dose, and then placed on the surfaces of NBA plates at 37°C for various times before selection as revertants. A single representative experiment.


PERCENTAGE OF THE MAXIMUM MUTATION YIELD :
0
10
20
30
40
50
60
70
80
90
100
MINUTES OF INCUBATION ON NBA BEFORE SELECTION OF MUTANTS :
0
20
40
60
80
100
120

experiments, a representative experiment being detailed in Table 22. The comparable experiments with EMS will be found in Tables 15 and 16. In all three Tables, mutation frequencies have been expressed for direct comparison as per 10^7 survivors. UV-induced mutation frequencies were 1-2 orders of magnitude less than those induced with EMS. Particularly noteworthy was the occurrence of a broth effect, the magnitude of which was greater than for EMS-induced mutation. As with EMS however, the full broth effect was not shown at the lowest doses. Essentially similar results have been demonstrated in the UV mutagenesis of E. coli B/r (Clarke 1967).

c. Results of the Filter Membrane Technique after UV.

Results of some experiments with this second technique are given in Table 23. Survival was in all cases above 80%, as measured by resuspending cells from membranes by vigorous shaking, diluting and plating. Comparison of Tables 22 and 23 show that mutant yield was 2-3 times greater with the filter membrane technique than by direct spreading (cf. typically, the responses to a 20-seconds dose of UV), with the added advantage that the larger populations used returned much greater numbers of induced mutants. The disadvantage is that a broth effect cannot be demonstrated in analogous manner to EMS and UV as with the direct spreading technique. It has proved possible to partially circumvent this difficulty by interposing posttreatments between irradiation and the 2-hour incubation on NBA (steps 2 and 3), i.e. before mutation fixation and expression can take place.

TABLE 22. The Induction of Supersuppressor Mutants in strain UV2 with UV, studied by the Direct Plating Technique. A Representative Experiment.

MUTANTS RECOVERED ON SELECTIVE AGAR PLUS:	UV DOSE IN SECONDS:				
	20	40	60	80	120
NO ADDITION	NO DETECTABLE DIFFERENCE FROM THE LEVEL OF SPONTANEOUS MUTATION				
1·5 µg/ml each of histidine and leucine	0·64 7	0·89 8	1·44 13	2·16 12	3·31 13
1·5% v/v NB	1·84 20	4·33 39	7·09 64	12·3 68	16·0 63
BROTH EFFECT	2·87	4·87	4·92	5·69	4·83
% SURVIVAL	79·6	65·7	65·7	40·5	28·7

The upper figure of each pair is the induced mutation frequency per 10^7 survivors. The lower figure of each pair is the number of induced mutants scored per mean of three plates at each dose. At 100% survival, $1·37 \times 10^8$ cells were inoculated per plate.

TABLE 23. The Induction of Supersuppressor Mutants in strain UV2 with UV estimated by the Filter Membrane Technique.

UV DOSE IN SECONDS:	MUTANTS PER 10^7 SURVIVORS IN EXPERIMENT NUMBER:			
	1	2	3	4
2	0·025	-	-	-
5	1·7	0·25	0·64	0·61
10	3·5	1·57	1·67	1·56
15	5·25	-	-	-
20	7·1	7·44	7·99	5·82

After UV, 2 hours on NBA, and 30 minutes starvation, mutants were recovered on unsupplemented selective agar. Each membrane received about 10^9 cells before UV, a constant number in each experiment. Each figure is derived from the mean of three membrane counts. Survival was in all cases better than 80%.

d. MFD after UV*

MFD was studied by holding irradiated cell populations on their carrier membranes on the surface of agar of desired composition before permitting the two-hour period on NBA. This period of fixation and expression was followed by selection of mutants, and mutation yields were then simply plotted against the time of interposed posttreatment.

Table 24 records the results of 5 such experiments with posttreatment on minimal agar plus glucose (MGA), containing none of the four requirements of the strain. Dramatic declines in mutation frequency are evident, these being almost completed in two hours of posttreatment. Resuspension of sample membranes showed that no corresponding decrease in survival occurred during this period. (It is regretted that the time to prepare membrane-borne populations is such that a greater number of sample times could not easily be studied in a single experiment, together with adequate controls. There is excellent agreement between the five experiments presented, however.).

For comparison with the results obtained with EMS, inasmuch as this is possible, MFD has been studied on other agars after the UV-induction of revertants. None of the following posttreatment agars showed any reduction of the MFD noted on MGA - unsupplemented selective agar, selective agar plus 1·5% v/v NB, or MGA with 30 μg/ml of each of the four growth requirements. UV-induction of supersuppressor

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* a short note of these results has been published in Microbial Genetics Bulletin 1967. No. 26. pp. 5-6.

TABLE 24. MFD of UV-induced Supersuppressor Mutants of B. subtilis strain UV2 as a function of Length of Posttreatment on MGA before Mutation Fixation and Expression on NBA.

LENGTH OF MGA POST-TREATMENT IN MINUTES:	% OF INITIAL MUTATION FREQUENCIES RECOVERABLE IN EXPERIMENTS:				
	1	2	3	4	5
0	100	100	100	100	100
30	-	23·9	18·2	24·6	24·1
60	13·64	-	-	-	-
75	-	11·1	11·3	11·5	10·4
120	8·39	12·0	7·4	11·8	9·4
180	8·74	-	-	-	-

Each figure is derived from the mean of three membrane counts. Each membrane received initially about 10^9 cells, a constant number in each experiment. A single dose of 15 seconds of UV was given to all membranes in each experiment, controls excepted. In all cases, survivals were better than 80%.

mutants thus differs from EMS-induction by the permission of MFD in the presence of 1·5% v/v NB. The requirement of greater quantities of enrichment for the prevention of MFD after UV compared to that after EMS almost certainly reflects the different sizes of plate (or membrane) inocula, about 10^9 for UV-induction versus about 10^7 for EMS-induction. As with EMS-induction however, there was no evidence of a sparing of MFD after UV-induction given by the inclusion of the four growth requirements of the strain (including leucine and uracil) in solid posttreatment agars.

SUMMARY OF UV-MUTAGENESIS AND UV-MFD: UV was a much less efficient mutagen for the induction of supersuppressor revertants of the his-A1 and leu markers than EMS. Few mutants were recovered by the same direct plating technique used for recovery of EMS-induced mutants, although medial dependence followed the same pattern for the two mutagens. The broth effect was larger for UV than for EMS, although similarly less than maximal at low doses of mutagen. More mutants were recovered with the filter membrane technique, especially when larger populations were irradiated. Comparison of medial dependence was not possible with the filter membrane technique, although posttreatments revealed a MFD. This MFD was not inhibited by any or all of the growth requirements of the strain: a similar result was previously found with EMS on solid media though not in liquid. Direct comparison of UV and EMS induction by the filter membrane technique is made in the next section.

8. A Direct Comparison of MFD after the Induction of Supersuppressor Revertants with EMS and UV.

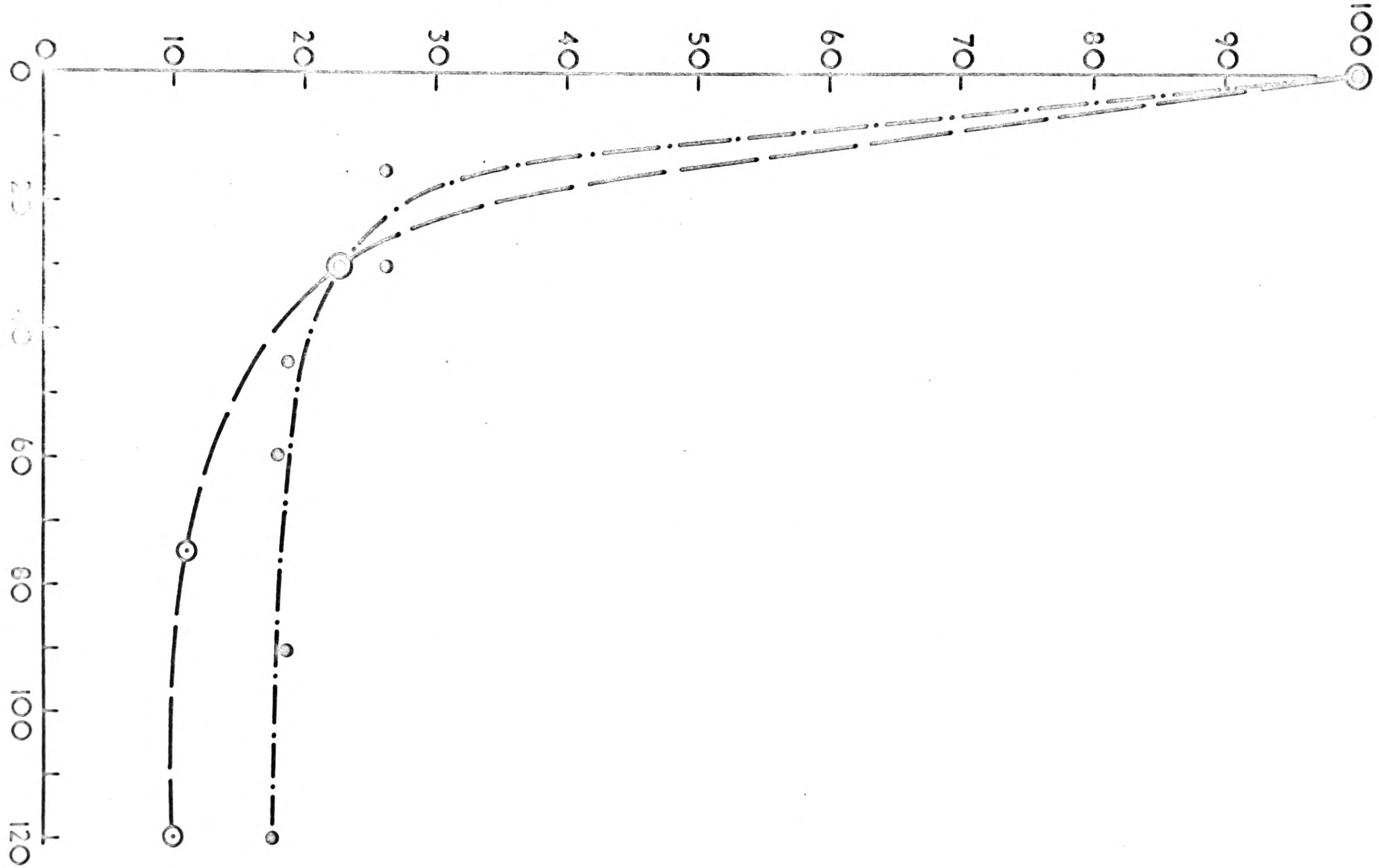
The modification of induced mutation frequencies after treatment with EMS and UV has been described, with a different mutant recovery technique used for each mutagen. It was therefore decided to study MFD after the EMS-induction of mutants by the same recovery technique as was used for UV-induction. Approximately 10^9 cells were plated per membrane after EMS-treatment (a constant number in each experiment) and the cells then incubated on NBA for 2 hours, starved for 30 minutes, and the mutants selected on unsupplemented selective agar. In the manner of the UV-experiments described in the preceding section, posttreatments were interposed between the initial application of cells to membranes and mutation fixation and expression on NBA. The ability of NBA incubation to return high mutation yields after EMS-induction has already been recorded in RESULTS section 6c. The dose of EMS normally applied was severely cut in these experiments to allow mutation induction to be studied with the larger populations: 15 minutes of 0·05 M. EMS being given at 37'C.

The results of three such experiments are shown in combination in Figure 8. For comparison, results of similar UV-experiments are also given on the graph (results of experiments 2-5 of Table 4), and the similar rates and extent of MFD after the two separate mutagen treatments are quite apparent. Further experiment has shown that, as with UV-MFD, no sparing of EMS-MFD was detected with this technique

FIGURE 8.

A comparison of MFD of supersuppressor reversion induced with UV (open symbols - broken line) or EMS (filled symbols - irregular line) on MGA with resuspension and plating on selective agar + 1·5% v/v nutrient broth. A single representative experiment.

PERCENTAGE OF MUTATION FREQUENCY RECOVERED ON IMMEDIATE PLATING ON BROTH-SUPPLEMENTED AGAR :



during posttreatment on unsupplemented selective agar, or on MGA with 30 µg/ml of each of the 4 growth requirements (including leucine and uracil). In addition, only very slight sparing of MFD was shown by selective agar plus 1·5% v/v NB, in decided contrast to its stimulatory effect on EMS-induced mutation yield described previously for different doses and techniques. This reduction in the effect of broth is almost certainly due to the 100-fold increase in cell density, the effects of such factors having been previously established (Table 19).

9. The Induction of Supersuppressor Mutants of strain UV2 with DEB, MMS, and NTG, and a Comparison of the Results of other workers with this last Mutagen.

The his-A1 marker was shown in Table 4 to be reverted in spot-tests by both DEB and NTG in addition to UV and EMS. It was of interest to determine whether revertants induced by such mutagens were also of the supersuppressor type, and if similar medial dependence of mutation yield occurred.

a. Mutation Induction with DEB.

The results of one (of three similar) experiment(s) with DEB and strain UV2 is shown in Table 25. DEB was used at a final concentration of 0·01 M, at 37°C, for various times, when the cells were washed and plated. Very few mutants were recovered on unsupplemented selective agar, their numbers not differing greatly from the spontaneous values. Few mutants were recovered on selective agar plus 1·5 µg/ml. each of histidine and leucine, although an increase of induced mutation

TABLE 25. The Induction of Supersuppressor Mutants with DEB and their Recovery on Different Media. - a representative experiment.

MUTANTS RECOVERED ON SELECTIVE AGAR PLUS:	TIME OF EXPOSURE OF CELLS TO DEB (0·01 M, 37'C) IN MINUTES:			
	15	30	45	60
NO ADDITION	NO SIGNIFICANT DIFFERENCE FROM SPONTANEOUS VALUES.			
1·5 µg/ml each of histidine and leucine	0·71 160	0·4 70	1·0 155	1·23 160
1·5% v/v NB	1·17 265	1·64 290	3·13 485	4·15 540
BROTH EFEECT	1·66	4·14	3·13	3·38

% Survival was in all cases better than 50%. The upper figure of each pair is the induced mutation frequency per 10^7 survivors. The lower figure of each pair is the number of induced mutants per ml of treated suspension, corrected for spontaneous values, and each figure is derived from the mean of three plate counts at each dose.

frequency became apparent at the higher doses. More mutants were recovered on broth-supplemented selective agar, thus demonstrating the broth effect for the induction of su^+ mutants in this system with DEB. The magnitude of this broth effect was much as for EMS-induction, as was its dose dependence - smallest at the lowest DEB dose.

The low mutagenicity of this low concentration of DEB (relative to those tolerated by other microorganisms) show that DEB is not a rewarding mutagen to study in this system: no further investigations were made.

b. Mutation Induction with MMS.

Studies of the effects of MMS on B. subtilis at the biochemical level have been carried out by Strauss and his coworkers (see Strauss, Reiter, and Searashi 1966), including the susceptibility to repair of lesions induced with this mutagen. In only one paper has the mutagenic effect of MMS on B. subtilis been reported (Strauss 1963), no attempt being made to study the medial dependence of mutation frequency induced in the met^- to met^+ system used. The induction of su^+ mutants in strain UV2 has shown that the agent is more lethal than higher concentrations of its ethyl counterpart, as previously reported for E. coli and its phages (see Loveless 1966 for review). Results obtained in two experiments with MMS are given in Table 26. The two experiments differ much more than is usual for replicate experiments with other chemical mutagens. A broth effect is evident, although its magnitude varied between the two

TABLE 26. The Induction of Supersuppressor Mutants with MMS and their Recovery on Different Media. - 2 experiments.

MUTANTS RECOVERED ON SELECTIVE AGAR PLUS:	TIME OF EXPOSURE OF CELLS TO MMS (0·025 M, 37'C) IN MINUTES IN EXPERIMENT NO.:					
	EXPERIMENT NUMBER 1.			EXPERIMENT NUMBER 2.		
	15	30	45	15	30	45
1·5 µg/ml each of histidine and leucine	1·3 260	9·5 1520	33·8 3310	0·89 80	4·66 210	22·7 650
1·5% v/v NB	4·2 840	27·3 4360	135·0 13230	7·28 650	61·4 2770	152·2 4370
BROTH EFFECT	3·23	2·87	3·99	8·2	13·2	6·72
SURVIVING CELLS PER ML. x 10^8	20	16	9·8	8·93	4·51	2·87

The upper figure of each pair is the induced mutation frequency per 10^7 survivors. The lower figure of each pair is the number of induced mutants scored per ml of treated suspension, calculated from plate counts and the dilution factor. Each figure is derived from the mean of three plate counts at each dose.

experiments, perhaps reflecting the differences in plating density and survival. As the ethyl methanesulphonate was mutagenically superior, both in terms of numbers of mutants recovered and of higher survivals, work with the methyl ester was discounted.

c. Mutation Induction with NTG.

In Table 27 are presented the results of one (of 3 essentially similar) dose response experiment(s) with NTG and strain UV2 of B. subtilis. It is apparent that NTG induced many su^+ mutants; indeed a concentration of 25 µg/ml yielded the greatest mutation frequencies ever recorded with this system. Perhaps the most striking observation was not that a broth effect was found, but its contrast with those reported for other mutagens. The NTG-broth effect was highest at the lowest doses of mutagen, reducing thereafter, in direct contrast to the broth effects found for EMS, DEB, and UV, which were smallest at the lowest doses increasing to relatively constant values thereafter. A similar decrease was also shown in the stimulatory effect of histidine and leucine in the agar, in this instance following the same pattern as for EMS, highest at low doses, lowest at low doses. As broth effects are a common property of all mutagens tried in this system, this anti-parallelism can only be explained as the interference by the mutagens to a greater or lesser extent with the pathways of mutation fixation and expression which give rise to the broth effects. Mutagens thus appear to be able not only to induce mutation, but also to actively modify the expression

TABLE 27. The Induction of Supersuppressor Mutants with NTG and their Recovery on Different Media. - a representative experiment.

MUTANTS RECOVERED ON SELECTIVE AGAR PLUS:	TIME OF EXPOSURE OF CELLS TO NTG (25 μg/ml, 37°C) IN MINUTES:				
	10	20	30	40	60
NO ADDITION	0·66 50	10·2 580	13·7 740	26·9 1210	49·7 1740
1·5 μg/ml each of histidine and leucine	30·3 2300	159·6 9100	246·3 13300	288·9 13000	337·0 11800
1·5% v/v NB	199·0 20700	475·5 44700	610·0 59800	817·7 54800	553·8 28800
BROTH EFFECT	6·58	2·98	2·48	2·83	1·64

Survivals for this experiment are given in Table 28. The upper figure of each pair is the induced mutation frequency per 10^7 survivors. The lowest figure of each pair is the number of induced mutants scored per ml of treated suspension, calculated from plate counts and the dilution factor. Each figure is derived from the mean of three plate counts at each dose.

of these mutations in a mutagen-dependent manner. It will be of interest in the future to attempt to divorce these two properties, e.g. DNA treated in vitro with one mutagen could be assayed for mutations by transformation. The recipient cells may be "modified" by treatment with the same or different mutagens, and the effect of this modification on the expression of the newly induced mutations studied.

MFD took place if NTG-treated cells were posttreated in a minimal liquid medium with glucose (Figure 9), although only for cells subsequently plated on broth-supplemented agar. The absence of an MFD for mutants recoverable on histidine and leucine-supplemented agar contrasts with the results found for EMS-mutagenesis. It should also be noted that the MFD for broth-plated cells was slightly reduced from that found with EMS and UV.

In contrast with results found with other mutagens, survival after NTG treatment was found to be medium-dependent. A representative experiment illustrates this in Table 28. At all NTG doses used, survivals were highest on selective agar plus 1·5% v/v NB, and lower on selective agar plus 1·5 μg/ml each of histidine and leucine, and on NBA. In general, the reduction in survival on these two media was dose-dependent. During posttreatment in liquid media, however, the counts on these two inferior media rose to the level found on broth-supplemented agar, as shown in the last line of Table 28.

The only other results published on quantitative NTG mutagenesis in B. subtilis are those of Yoshida and Yuki

FIGURE 9.

MFD of supersuppressor reversion induced by NTG. Mutation yield with increasing time of posttreatment in liquid minimal medium + glucose, plated on selective agar + 1·5% v/v nutrient broth (crosses) or + 1·5 μg/ml each of histidine and leucine (open triangles). A single representative experiment.

PERCENTAGE OF MUTATION FREQUENCY RECOVERED ON IMMEDIATE PLATING :

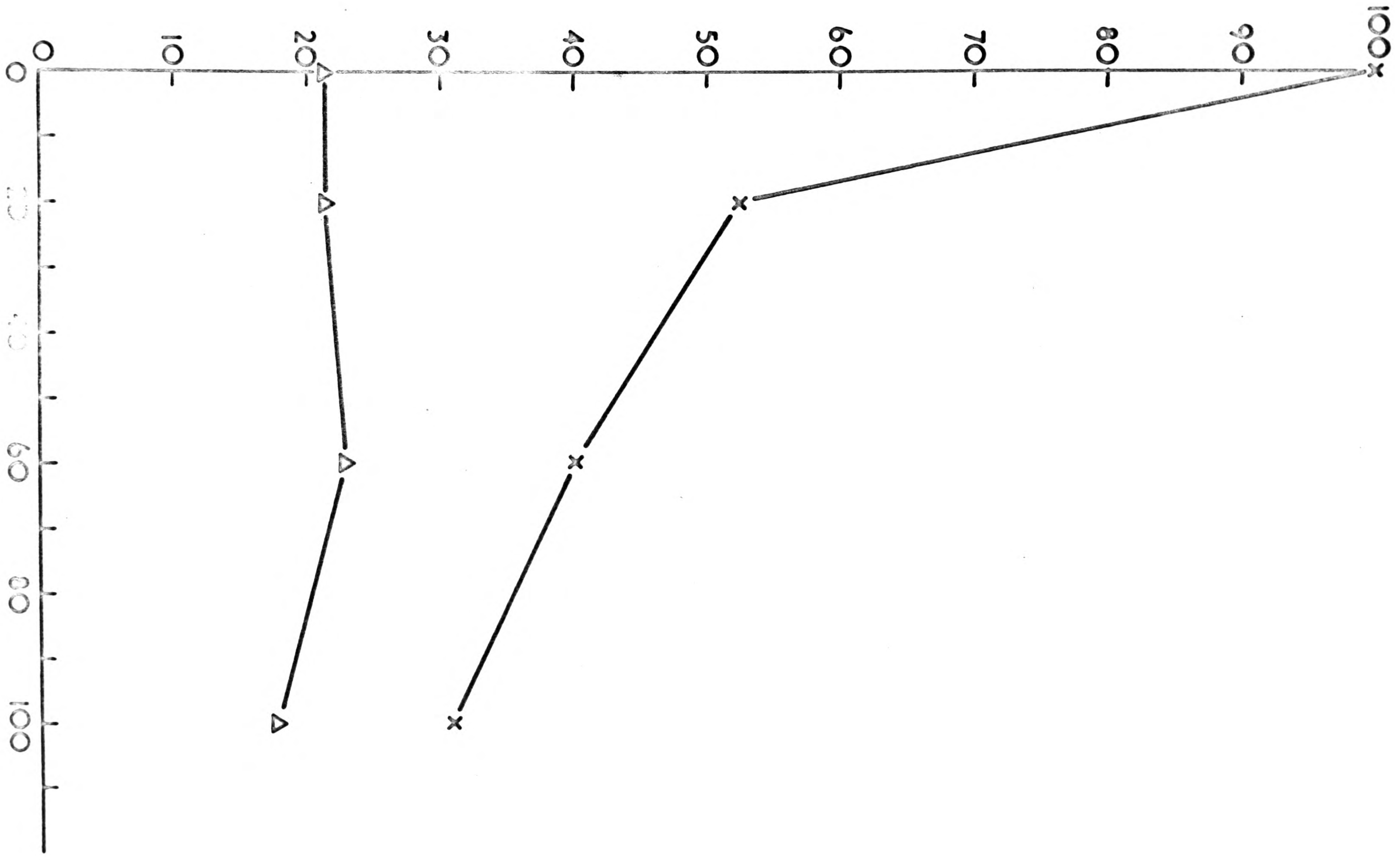


TABLE 28. Survival of NTG-treated cells of strain UV2 scored on agars of different composition. - A Representative Experiment.

DOSE OF NTG IN MINUTES AT 25 µg/ml 37'C.	SURVIVING CELLS PER ML x 10^9 ON:		
	Selective agar + 1·5% v/v NB.	Selective agar + 1·5 µg/ml histidine and leucine.	Nutrient Broth Agar
I.			
0	1·04	0·99	1·00
10	1·04	0·76	0·72
20	0·94	0·57	0·64
30	0·98	0·54	0·52
40	0·67	0·45	0·36
60	0·52	0·35	0·25
II.			
20	0·91	0·95	0·92

I = plated immediately after treatment, washing, and dilution.

II = plated as in I, but with 100 minutes posttreatment in liquid minimal salts plus glucose at 37'C between washing and plating.

Each figure is derived from the mean of three plate counts.

(1968). The discrepancies between their results and the results presented here are such that a detailed comparison is in order, paying special attention to the plating media used in each instance.*

1. In contrast to the supersuppressor system used in this work, the Japanese authors used a $phe^{-}try^{-}$ diauxotroph (also amylaseless), studying reversion of the leaky phe^{-} marker. After various times of treatment with the same concentration of NTG as used here, 25 µg/ml, they recovered mutation yields on unsupplemented selective agar comparable to the highest reported here on broth-supplemented agar. As there is a great difference in the yield of supersuppressors on these two medial types (Table 27), it is highly likely that after allowance is made for marker differences, the different levels of response to NTG shown by phe^{+} and su^{+} mutants on unsupplemented agar were due to the leakiness of the phe^{-} mutation. Leakiness may well allow as much residual synthesis and/or division as a trace of the previous growth requirement(s) of the strain, thus rendering phe^{+} mutation frequencies on unsupplemented agar more equivalent to those of su^{+} mutation frequencies on histidine and leucine-supplemented agar. There is no evidence of a leakiness of the su^{-} condition in strain UV2.

2. Yoshida and Yuki described an increase in survival of treated cell populations when they were posttreated in liquid minimal medium before plating. This increase in survival, albeit small, was attributed to the repair of

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* a cautiously worded statement of these discrepant results was published in Microbial Genetics Bulletin 1969. No. 30. pp. 8-9. (Corran J.).

lethal lesions caused by NTG. Survival was scored by these authors on NBA. An increase in "survival" was also detected in experiments reported here (Table 28), but in medium-dependent fashion. The last line of Table 28 demonstrates that holding treated cells in a liquid minimal medium for a time allowed counts to rise (scored on the two 'inferior' meda) to the level of the count on broth-supplemented minimal agar, this having remained relatively constant throughout. One of these 'inferior' media was NBA - the same plating medium used by Yoshida and Yuki. Although one cannot exclude a repair, it is much more probable that this rise in count on the two 'inferior' media represents the adaptation of NTG-treated cells to suboptimal plating media. Such medium effects are well known with, for example, E. coli B. That Yoshida and Yuki showed that their rise in survival was inhibited by acriflavine in no way constitutes proof of a recovery mechanism: this class of compound is well known to produce a multiplicity of deleterious effects, including binding to tRNA (Grosjean, Wérenne and Chantrenne 1968), inhibition of protein synthesis (Weinstein and Finkelstein 1967, Wérenne 1967), and of cytochrome synthesis (Nagao and Sugimura 1965) and prolongation of the lag phase of irradiated bacteria (Shankel and Kleinberg 1967).

3. The "repair" demonstrated by Yoshida and Yuki was claimed to affect only survival, as no decrease in mutation yield took place during the same posttreatment. It is quite clear from Figure 9 that MFD occurred of su^+ mutants recovered on broth-supplemented agar, although not of those

mutants recoverable on a lesser supplemented agar. The MFD can be demonstrated dependent on the plating medium, and it is thus probable that the Japanese workers did not observe it because they used a plating medium upon which one would not expect to describe MFD, minimal agar without supplement.

A second, but much less likely, explanation may lie with the induction in the two systems of different types of mutations. If the phe^+ revertants are true revertants, in comparison with the su^+ revertants reported here, then these two classes of mutation may well show different responses to posttreatments, as indeed has been claimed for E. coli WP2 try^- (Bridges, Dennis, and Munson 1967). Dr's Yoshida and Yuki have recently donated a culture of their strain, so it should soon be possible to totally resolve the discrepancies.

The most likely explanation, however, is still the use of inadequate plating medium in each instance by the Japanese authors.

SUMMARY OF RESULTS WITH CHEMICAL MUTAGENS OTHER THAN EMS: Low frequencies of su^+ reversion were induced by DEB and MMS, and very much higher frequencies by NTG. In all cases a broth effect was evident, the dose dependence of this stimulation being mutagen-dependent. The pattern of histidine and leucine effects for NTG parallels that for EMS, being highest at low doses, reducing thereafter. The pattern of broth effect for DEB parallels that for EMS, being

lowest at low doses and increasing thereafter to relatively constant values. The NTG broth effect in contrast was highest at low doses, decreasing thereafter.

Survival after DEB and MMS was similar on the different plating media used to score it, as for EMS, although both mutagens were appreciably more lethal than EMS at the doses used. In contrast, survival after NTG was medium-dependent, although the differences were abolished during posttreatment. MFD took place during posttreatments after NTG, although only (if strongly) of mutants recoverable on broth-supplemented agar. MFD for DEB and MMS has not been tested, although it may be possible to infer it from the presence of a broth effect on both cases.

10. Interacting Effects of Combined Mutagen Treatments.

Interaction between mutagenic treatments has been previously reported to cause two classes of effect:

1. On Lethality of the Treatments: interaction treatments are usually synergistic in that exposure to one mutagen "sensitises" the cells to a subsequent exposure to the same mutagen (dose fractionation) or different mutagens. Such interactions causing the enhancement of lethality have been reported for nitrogen mustard, UV, and X rays in E. coli B/r and S. cerevisiae (Haynes and Inch 1963, Haynes 1964). In the latter organism however, response to posttreatment after X rays was dependent on ploidy. Synergism between gamma and UV irradiations has been more recently demonstrated by Davies, Arlett, Munson,

and Bridges (1967) in E. coli B/r, and between DEB and UV in S. cerevisiae (Kilbey 1969). Saturation or inactivation of the dark repair mechanisms by the first mutagen has been put forward in explanation (Haynes 1964, Baptist, Haynes and Uretz 1966, Davies et al. 1967). The occurrence of synergism was independent of the order of mutagen treatments, although the degree of synergism often depended on the choice of primary mutagen. The pattern of photorepair is also altered in one system (Kilbey 1969).

There is only one report of an antagonism between mutagenic treatments - the alleviation of nitrogen mustard-induced lethality in Penicillium chrysogenum by manganous chloride (Morpurgo and Sermonti 1959).

2. On Mutagenicity of the Treatments: co mbined mutagen treatments have been reported synergistic for mutation induction as well as for lethality (e.g. Swanson 1952, Auerbach 1965, Davies et al., 1967, Bridges, Munson, Arlett, and Davies 1967). This synergism was manifested as a mutant recovery after the combined treatments greater than could be attributed to the two component treatments given separately. An exception was the absence of synergism in the combined UV and X ray induction of high level streptomycin resistent mutants in E. coli B/r (Doneson and Shankel 1964): even the simple additivity of the two treatments disappeared at the higher X ray doses, suggesting a selective inactivation of mutants. Similar results for UV and X rays have been recorded in the ad^- $inos^-$ system of Neurospora crassa (Auerbach - unpublished).

In the report by Bridges et al. (1967), the interaction mutants were found to behave as if they had been induced by the UV component rather than the gamma ray component. A specificity of interaction treatments has been shown by Auerbach (in preparation) in Neurospora crassa with UV and DEB or HNO_2. Pretreatment of conidia with DEB in increasing doses led to the subsequent induction by a standard dose of UV of increasing numbers of adenine reversions and decreasing numbers of inositol reversions in an ad^- $inos^-$ diauxotroph (expressed per survivors). It was accordingly of interest to test for interaction in B. subtilis. These experiments were carried before it was discovered that induced his-A1 revertants were largely supersuppressor mutants: mutation was therefore scored as histidine reversion. As in previous UV experiments, low doses were used in order to minimise induction of defective lysogens.

a. Possible Interaction between UV and DEB.

Strain SB1 $his\text{-}A1^-$ $try\text{-}2^-$ was treated with various doses of DEB followed by small doses of UV. Induced mutants were then recovered as UV-induced revertants by the filter membrane technique. Results of two of the six experiments performed are shown in Table 29, and it may be clearly seen that in these instances prior DEB treatment did not lead to the subsequent induction by UV of a greater frequency of mutation than by UV alone. Of the four experiments not given in the Table, three showed a slight reduction in UV-induced mutation after DEB pretreatment, and one a small increase. There is therefore no evidence for the

TABLE 29. Induction of his^+ Revertants in strain SB1 by DEB and UV in combination, - the UV induction of these revertants in cells previously treated with DEB. - 2 experiments.

NOTE:- The figures in the main part of the Table represent UV-induced mutants per 10^8 survivors. Correction has been made by subtracting, at each dose, the calculated survival of spontaneous and DEB-induced revertants. It has been assumed that survival of these mutants is as of the bulk of the population, although this has not been experimentally verified.

UV DOSE IN SECONDS:	MUTANTS PER 10^8 SURVIVORS, INDUCED BY UV IN: Experiment 1.			Experiment 2.		
	DEB DOSE IN MINUTES (0·01 M, 37°C)					
	0	20	45	0	30	60
0	0	0	0	0	0	0
2	5·0	4·1	3·0	5·3	4·1	5·8
5	11·3	8·9	8·8	17·0	13·9	9·6
10	28·4	19·1	29·4	44·4	45·9	40·4
20	47·1	54·3	54·4	114·3	111·0	126·8
% SURVIVALS AFTER DEB ALONE	100	31·85	16·6	100	53·75	16·25
DEB INDUCED MUTANTS PER 10^8 SURVIVORS	0	6·14	42·24	0	33·4	95·2

Each figure is derived from the mean of three membrane counts. All UV doses gave c. 100% survival.

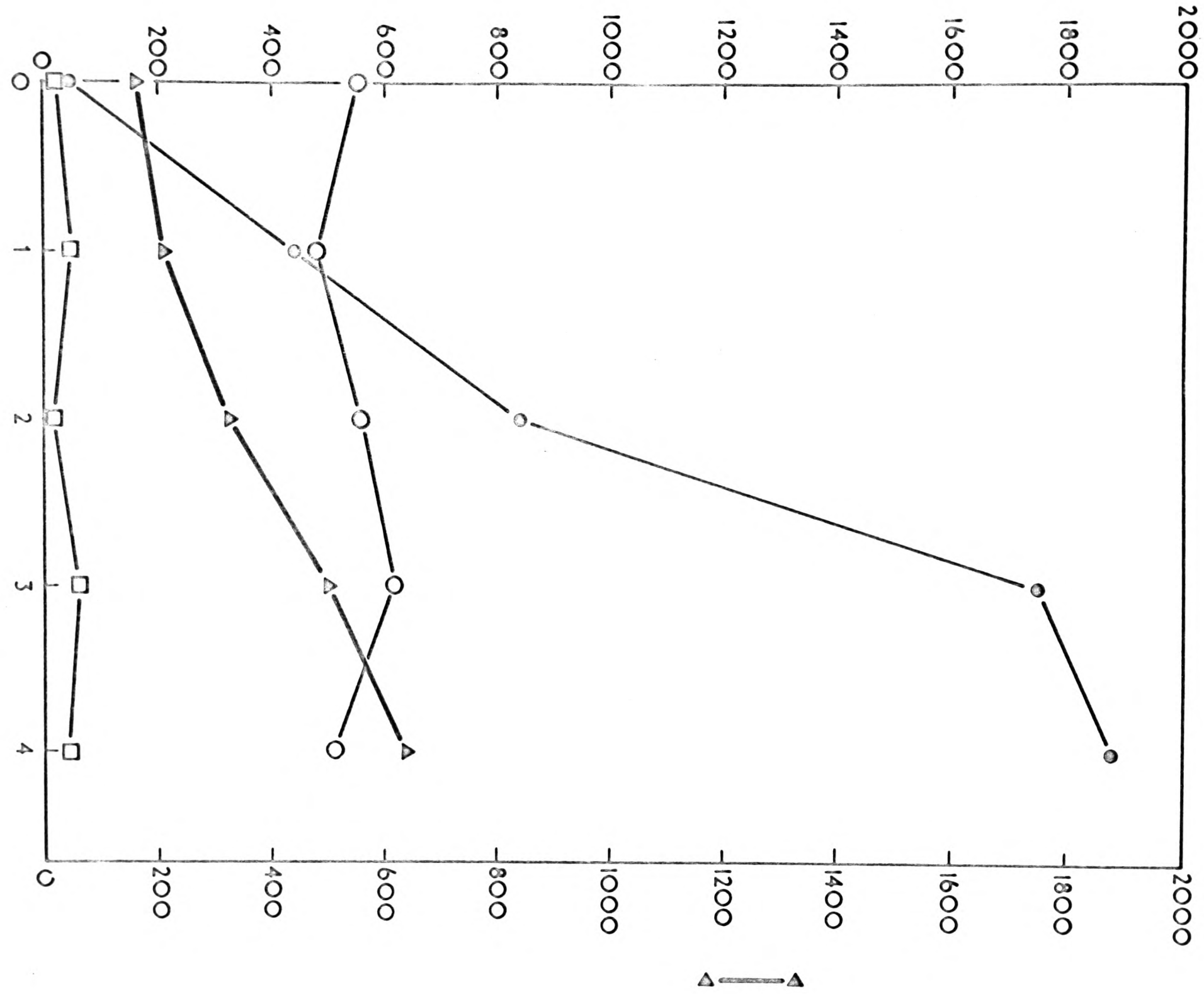
existence of synergism between a range of DEB doses and very low UV doses in this strain. The UV doses used in these experiments gave in all cases survival indistinguishable from 100%, even after a DEB dose inactivating 86% of cells treated.

b. Possible Interaction between UV and 2AP.

In Table 4 it is recorded that whereas the ura-1 marker reverted in spot-tests with both UV and 2AP, the his-A1 marker responded only to UV. It was therefore of interest to look for interaction between these two mutagens with the two markers. The base analogue cannot be expected to interfere with repair mechanisms in the manner postulated for EMS (Haynes 1966) or DEB (Kilbey 1969). Nevertheless, the mutational changes brought about by 2 AP might provide substrate for these recovery mechanisms and divert their attention from subsequently induced UV damage. This hypothesis has been recently reinforced by the finding that a dark repairless mutant of E. coli K12 (together with its partial revertant to wild type) was more mutable with 2AP than its wild type parent (Smirnov and Skavronskaya 1969).

Mutability of the markers of strain SB5 his-A1$^-$ ura-1$^-$ try-2$^-$ by 2AP was then tested. The results of one (of four essentially similar) experiment(s) is shown in Figure 10. In comparison with the increase in total cell count over the four hours of incubation in 200 µg/ml 2AP, his$^+$ and try$^+$ revertants showed no increase. Indeed their relatively constant number suggests that they arose not in the liquid culture but on the mutant recovery plates. Ura$^+$ revertants

REVERTANTS PER ML. OF CULTURE :



TOTAL VIABLE CELLS PER ML. OF CULTURE x 10^6 .

on the other hand increased at a much faster rate than the total population, particularly during the initial period of mutant appearance, signifying their induction by the 2AP. Few revertants of any kind were recovered without 2AP, and it was shown by comparison with control samples that 2AP did not reduce growth rate to any measurable degree.

Interaction experiments were then performed with 2AP and UV, the results of one (of four essentially similar) experiment(s) being recorded in Table 30. 2AP treatments over 90 minutes had no effect on subsequent killing or mutation induction by UV at the very low doses used. UV-induced histidine and uracil reversions were very similar in 2AP-treated or untreated cells. The UV doses used gave survivals not detectably different from 100%. It may be concluded that prior 2 AP treatment has no synergistic interaction with the induction of mutation by very low doses of UV in this strain.

In both types of attempted interaction experiment, no such synergism has been found with 2AP or DEB and UV. When the B. subtilis strains have been "cured" of their defective lysogens, an imminent event, it would be instructive to repeat these experiments with higher UV doses.

11. Broth Effects on Transformation and Transduction.

Broth effects have been demonstrated for the recovery of supersuppressor mutants of B. subtilis after treatment with a number of mutagens. It has been further shown that this action of broth is upon the pathways of fixation and

TABLE 30. Induction of his^+ and ura^+ revertants in strain BD2 (SB5) of B. subtilis by 2AP and UV in combination - the UV induction of these revertants in cells previously treated with 2AP - a representative experiment.

NOTE:- as in Table 29, the figures in the main part of the Table represent UV-induced mutants per 10^8 survivors, with the calculated survival of spontaneous and 2AP-induced mutants (if any) subtracted at each dose. Assumption has been made that survival of these mutants is as of the bulk of the population.

UV DOSE IN SECONDS:	MUTANTS PER 10^8 SURVIVORS INDUCED BY UV:					
	URACIL REVERTANTS			HISTIDINE REVERTANTS		
	UV only no 2AP.	2AP followed by UV.	∴ UV induced in 2AP'd cells	UV only no 2AP	2AP followed by UV.	∴ UV induced in 2AP'd cells
0	0	261	0	0	0	0
5	79	325	74	80	76	76
15	247	492	231	175	183	183

Each figure is derived from the mean of three membrane counts. UV survival was in all cases indistinguishable from 100%.

expression of these mutations. The efficiency with which a su^+ character newly introduced to the cell by mutation is established is thus enhanced by the presence of broth. It is of great interest to determine whether a broth effect is characteristic of the introduction of a new marker per se, or only of its introduction by mutation. In the E. coli WP2 system, the existence of a broth effect has been claimed to be both mutagen- and mutation-specific (Bridges, Dennis, and Munson 1967, Bridges - personal communication), at least for UV and X rays. This argues against the broth effect being a general property of the introduction of new markers, although it is possible that particular mutagens may reduce or increase a general broth effect by secondary action. Unfortunately no comparable experiments have been performed (or reported) in the strain WP2 after the introduction of the same wild-type revertant and supersuppressor mutations by transduction. In contrast, Witkin (1956) has reported a great difference in the degree of broth requirement for the establishment of try^+, ad^+, and lys^+ prototrophy in S. typhimurium dependent on whether the markers were introduced by transduction or mutation. In all cases, a broth effect was reported, although much more broth was required after UV mutagenesis.

Experiments were therefore performed in which strain UV2 of B. subtilis was transformed or transduced to joint independence of histidine and leucine with supersuppressor or wild-type markers.

a. Transformation.

Tables 31 and 32 record the results of two experiments performed (amongst others) in which a transformation broth effect was sought. Joint independence of histidine and leucine was introduced to the recipient strain in one of two ways, either with su^+ DNA, or by double transformation of the his^+ and leu^+ markers with saturating concentrations of wild-type DNA. It will be seen that a broth effect on transformation occurred regardless of the source of joint independence of histidine and leucine. In addition, the variable results in the two experiments demonstrate that the su^+ character does not in general show a greater broth requirement than the wild-type markers. A broth effect was also shown (Table 31) for each of three independent wild-type markers. It may be concluded that broth has a generally stimulatory effect on the incorporation of transformed markers. The broth effect is not upon DNA uptake as this step in the procedure was terminated with DNAase before the cells were exposed to broth in the plating medium.

As broth has been demonstrated to yield increased frequencies of induced supersuppressor revertants in this strain of B. subtilis, the necessary complement to these experiments is the study of the induction of true wild-type revertants. With this in mind, some of the markers detailed in Tables 5-8 have been further examined, although none of them have as yet given evidence of true reversion in a definite manner, the procedures being in any case laborious. This investigation will be continued.

TABLE 31. The Response to Medial Supplementation of Transformation Yield in strain UV2. - a representative experiment.

TRANSFORMANTS SCORED ON SELECTIVE AGAR PLUS:	TRANSFORMANTS PER 1 ML. ALIQUOT OF RECIPIENT CULTURE:*				
	to SU^+	to HIS^+ LEU^+	to LEU^+	to HIS^+	to TRY^+
NO ADDITION	3200	100	14,000	22,000	6,000
1·5 µg/ml EACH OF HISTIDINE AND LEUCINE	2300	100	14,000	35,000	6,500
1·5% v/v NB	5900	200	28,000	62,000	19,000
DNA SOURCE	su^+	wild-type	wild-type	wild-type	wild-type
BROTH EFFECT	2·57	2·0	2·0	1·77	2·92

* Calculated from plate counts and the dilution factor. Each figure is derived from the mean of three plate counts. Wild-type DNA was used at saturating concentrations to yield as many his^+ leu^+ transformants as possible.

TABLE 32. The Response to Medial Supplementation of Transformation to Independence of Histidine and Leucine in strain UV2. - a second representative experiment.

TRANSFORMANTS SCORED ON SELECTIVE AGAR PLUS:	TRANSFORMANTS PER 1 ML. ALIQUOT OF RECIPIENT CULTURE*	
	to SU^+	to HIS^+ LEU^+
NO ADDITION	336,000	26,800
1·5 µg/ml each of HISTIDINE and LEUCINE	350,000	24,500
1·5% v/v NB	505,000	56,200
DNA SOURCE	su^+	wild-type
BROTH EFFECT	1·44	2·29

* Calculated from plate counts and the dilution factor. Each figure is derived from the mean of three plate counts. Wild-type DNA was used at saturating concentrations to yield as many his^+ leu^+ double transformants as possible.

The results of Tables 31 and 32 are similar in essence to those of Jensen and Haas (1962). Using another strain of B. subtilis, they also reported no increase in transformation on selective media supplemented with traces of previous growth requirement. Transformation frequency increased however when DNA-treated recipient cells were incubated on a complete medium (NBA) for various times before selection. This last result is presumably analogous to the stimulation of transformation yield by broth reported here.

b. Transduction.

The results of comparable transduction experiments with phage PBS1 serve only to complicate an already complex phenomenon (Table 33). It will be seen that supplementation of the selective media for a number of markers produced entirely variable results in different experiments. These included both a reduction and an increase in transductant recovery of different markers in the same or different experiments. In addition, no su^+ transductant was ever recovered on unsupplemented selective agar, a finding which brings to mind the requirement of minute quantities of previous requirement before the establishment of phenotypic suppression by antibiotics (Kirschmann and Davis 1969). Their appearance on the same selective medium after transformation presumably reflects an interesting difference in the metabolic states of recipient cells for both types of genetic transfer. This difference will be examined in the future as the mutability of cells may similarly differ.

TABLE 33. The Response to Medial Supplementation of Transduction Yield in strain UV2.

TRANSDUCTANTS RECOVERED ON SELECTIVE AGAR PLUS:	TRANSDUCTANTS PER 1 ML. ALIQUOT OF RECIPIENT CULTURE* TO											
	LEU^+ IN experiment no.			HIS^+ IN experiment no.			TRY^+ IN experiment no.				SU^+ IN experiment no.	
	1	2	3	1	2	3	1	2	3	4	1	2
NO ADDITION	810	210	3410	1450	420	5880	370	120	2180	60	0	0
1·5 µg/ml EACH OF HISTIDINE AND LEUCINE	830	220	2510	1160	380	3070	490	80	1910	70	290	470
1·5% v/v NB	570	300	1160	1130	390	2030	760	250	1340	150	410	250

* calculated from plate counts and the dilution factor. Each figure in the Table is derived from three plate counts.
Note: no double his^+ leu^+ transductants were ever recovered.

Great discrepancies of the kind reported here have also been cited by Csiszar and Ivanovics (1965) with phage 3NT transduction of other strains of B. subtilis. Two plating media were used by these authors, a minimal salts medium plus glucose, and minimal salts medium plus glycerol and glutamic acid. Aliquots of phage-treated recipient cells yielded numbers of transductants which varied greatly on the two media dependent on the growth phase of the culture, the age of the phage preparation, and the multiplicity of infection. No explanation was offered for this extraordinary phenomenon, which is worthy of investigation in its own right.

12. The Analysis of an apparent case of "Gene-Controlled Mutational Stability".*

Results in this section are presented in terms of the interaction between the histidine and threonine requirements of strain UV2, his-A1$^-$ leu$^-$ ura-1$^-$ try-2$^-$, either threo$^+$ or with the threo$^-$ marker of strain AP3. The other requirements, for uracil, leucine, and tryptophan, will be disregarded here, being, unless otherwise stated, constantly satisfied with 30 µg/ml of each substance.

Table 11 (RESULTS section 4) records an apparent effect of the threo (AP3) marker on the spontaneous reversion of the his-A1 marker. In a threo$^+$ background, histidine revertants were produced in numbers independent of the initial plating density, provided that the plating medium was capable

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* Published in Mutation Res. 1969. 7 (3). 287-295. (Corran J.).

of sustaining some residual division (Table 10). Introduction of the threo marker into the strain led to the recovery of very few histidine revertants: in the representative experiment given in Table 34, none were in fact recovered. This Table also records that the "inhibition" of histidine revertants was not caused by the addition to the selective agar of the additionally required amino acid, threonine, as the threo^{+} strain produced histidine revertants regardless of this amino acid. It would appear that the threonine requirement rather than the amino acid itself is responsible for the paucity of histidine revertants.

The situation is so far strikingly reminiscent of the phenomenon of "gene-controlled mutational stability" reported for the try-6 marker of E. coli B/r WP2 on the addition of a particular adenine requirement (Chopra 1967). In this E. coli system, no tryptophan revertants were ever recovered in the presence of the second auxotrophy. In contrast, the threo^{-} his-Al^{-} strain of B. subtilis irregularly yielded small numbers of histidine revertants in some experiments. In addition, variable numbers of his^{+} revertants were recovered (as a general rule) when the trace supplementation of 1·0 μg/ml histidine in the selective agar was replaced by 1·0 - 1·5% v/v NB. The production of some his^{+} revertants in the presence of the threo marker presumably shows that the pathway to histidine reversion is not completely blocked, as the equivalent pathway appears to be in the E. coli system. The effect may thus be due to a selection against histidine revertants in a threo^{-} background. The production of

TABLE 34. Number of Spontaneous his^+ Revertants derived from his^- $threo^+$ and his^- $threo^-$ Substrains of UV2 in a Typical Experiment.

SUBSTRAIN OF UV2.	NUMBER OF CELLS INOCULATED PER PLATE	HIS^+ MUTANTS (MEAN OF 10 PLATES) ARISING ON SELECTIVE MEDIUM + 1 μg/ml HISTIDINE	
		Without added threonine.	With 30 μg/ml threonine.
his^- $threo^+$	$4{\cdot}9 \times 10^7$	128	151
	$4{\cdot}9 \times 10^6$	124	114
his^- $threo^-$	$7{\cdot}0 \times 10^7$	-	0
	$7{\cdot}0 \times 10^6$	-	0

variable numbers of mutants on parallel plates has also been reported (Grigg 1958) in a study of selection against revertants attributable to the exhaustion of the medial energy source.

The influence of background growth was then examined for a selective action against revertants by varying the trace concentrations of histidine and/or broth added to the selective agar. The results of such an experiment given in Table 35 show quite clearly that histidine revertants arose in the his^- $threo^+$ strain, their numbers increasing with the concentration of added trace supplements. Once again, mutant recovery was relatively independent of initial plating density. A similar phenomenon has been reported with a strain of E. coli by Mohn (1968), where the yield of spontaneous revertants of a met^- marker increased with the broth supplementation of the selective agar. In both cases, the number of spontaneous mutants observed depends on the final population size achieved on the plates. This in turn depends on the amount of residual growth permitted to the inoculum by trace supplementation of the agar.

In contrast, however, an increase in revertant number occurred in the his^- $threo^-$ strain only at low concentrations of supplement. As these concentrations of supplement increased, a selection against revertants became evident, being more marked on histidine-supplemented agar than on a broth supplement. In the latter case, selection was also influenced by plating density, being more marked with larger

TABLE 35. A Representative Experiment showing the Effect of Histidine and/or Nutrient Broth Concentration on the Yield of his^+ Revertants from his^- $threo^+$ and his^- $threo^-$ Substrains of UV2 on Selective Agar plus 30 µg/ml each of Leucine, Uracil, Tryptophan, and Threonine.

CONCENTRATIONS OF ADDED SUPPLEMENT		SUBSTRAIN AND INOCULUM OF CELLS ADDED PER PLATE: YIELD OF his^+ REVERTANTS			
% v/v NUTRIENT BROTH	µg/ml HISTIDINE	his^-threo^+		his^-threo^-	
		$2{\cdot}2 \times 10^7$	$2{\cdot}2 \times 10^6$	$2{\cdot}9 \times 10^7$	$2{\cdot}9 \times 10^6$
-	-	14	0	22	2
0·1	-	20	5	35	6
0·25	-	21	9	45	11
0·5	-	70	26	32	30
1·0	-	74	77	24*	80*
1·5	-	99	166	19*	51*
-	0·1	43	15	44	25
-	0·25	48	37	59	49
-	0·5	80	67	38*	43*
-	1·0	151	121	5*	4*
-	1·5	99	84	9*	18*

(Contd.)

TABLE 35 (Contd.)

CONCENTRATIONS OF ADDED SUPPLEMENT		SUBSTRAIN AND INOCULUM OF CELLS ADDED PER PLATE: YIELD OF his^+ REVERTANTS			
% v/v NUTRIENT BROTH	µg/ml HISTIDINE	his^-threo^+		his^-threo^-	
		$2{\cdot}2 \times 10^7$	$2{\cdot}2 \times 10^6$	$2{\cdot}9 \times 10^7$	$2{\cdot}9 \times 10^6$
0·5	0·5	101	124	13*	23*
1·0	0·5	160	132	2*	23*
0·5	1·0	91	136	0	5*
0·25	0·25	72	62	29*	25*

* denotes colonies very much reduced in size. Each figure represents the mean of three plate counts.

inocula. In addition, revertant colonies were considerably diminished in size at supplement concentrations above 1·0% v/v NB or 0·5 μg/ml histidine, or with all mixtures tried of broth and histidine. The appearance of selection against revertants only in the his^- $threo^-$ strain suggested that the threonine requirement was responsible, the strains being isogenic for all but the threo alleles. The background growth may, for example, exhaust the medial threonine, thus inhibiting the subsequent growth of his^+ but $threo^-$ revertants.

This hypothesis was tested in two ways:

1. Reconstruction experiments were performed to determine whether the genotype of histidine revertants with respect to threonine affected selection against them. In a number of experiments, plates of selective agar containing 30 μg threonine and 1 μg histidine per ml were spread with aliquots of his^- $threo^-$ cells and incubated at 37°C. Immediately, or after 1, 2, or 3 days of incubation, small numbers of his^+ $threo^-$ or his^+ $threo^+$ cells were spread on top of the background growth, and the plates then reincubated to allow any growth of these histidine prototrophs. The result of one such experiment is given in Table 36. It may be clearly seen that whereas both types of histidine prototroph grew if added at the same time as the his^- $threo^-$ background (0 hours, columns A, B, D, E.), only his^+ $threo^+$ cells grew when added thereafter (columns A and D), his^+ $threo^-$ cells being inhibited (columns B and E). It is evident that the discrimination

TABLE 36. A Representative Reconstruction Experiment: the Ability of Selective Medium + 30 μg Threonine and 1 μg Histidine per ml. to support the Growth of a Number of his^+ $threo^-$ or his^+ $threo^+$ Cells added at Various Times after the Inoculation of $1{\cdot}1 \times 10^7$ his^-threo^- Cells per Plate.

Time (hours) of inoculation of histidine prototrophs after seeding of plates with his^- $threo^-$ background and incubation at 37°C.	His^+ COLONIES PER MEAN OF 5 PLATES:				
	A Number of his^+ $threo^+$ cells added*	B Number of his^+ $threo^-$ cells added*	C his^-threo^- background only	D his^-threo^- background + added his^+ $threo^+$ cells	E his^-threo^- background + added his^+ $threo^-$ cells
0	152	125	3	126	115
24	231	211	0	255	0
48	241	76	0	243	0
72	395	256	0	454	0

* scored on selective medium + 30 μg threonine and 1 μg histidine per ml without added background.

against his$^+$ threo$^-$ cells by the his$^-$ threo$^-$ background was exerted, not immediately, but during and after the first 24 hours of incubation. This makes it probable that the only histidine revertants to be recovered from a his$^-$ threo$^-$ background will be those arising before this discrimination becomes effective. This would account for the small numbers of such revertants (if any) found on selective agar plus 30 µg threonine and 1 µg histidine per ml. The lack of discrimination against his$^+$ threo$^+$ cells shown in these experiments is strong support for the postulated explanation by exhaustion of medial threonine by the his$^-$ threo$^-$ background. In passing, it should be noted that the behaviour of histidine prototrophs in these reconstruction experiments was identical whether they had been recovered as histidine revertants or as transformants to histidine prototrophy with wild-type DNA.

Directly comparable experiments with a his$^-$ threo$^+$ (strain UV2) background could not be performed, as this strain gives rise to large numbers of histidine revertants (Table 10). Addition and spreading of a daily inoculum of histidine prototrophs would simply redistribute revertant colonies arising on the plates, and thus render scoring impossible. A technique was therefore devised whereby backgrounds of his$^-$ threo$^-$ or of his$^-$ threo$^+$ cells were incorporated <u>into</u> the selective agar (with 30 µg threonine and 1 µg histidine per ml.) and covered when solidified with a thin sterile layer of the same agar. The daily ration of histidine prototrophs was then spread on top of this agar layer as required.

Although it took at least twice as long for discrimination against revertants to become apparent when the background was in rather than on the agar, the pattern of inhibition was the same, where comparable. The experiments showed that his^+ $threo^-$ cells, but not his^+ $threo^+$, were inhibited by a his^- $threo^-$, but not by a his^- $threo^+$, background. This further strengthens the suggestion that discrimination was due to the exhaustion of medial threonine.

2. If the discrimination against histidine revertants was brought about by the preemption of medial threonine by the his^- $threo^-$ background, it should then be nullified by the addition of threonine in sufficient excess. The results of 5 experiments given in Table 37 conclusively demonstrate that this was so: at 300 μg/ml of threonine, histidine revertants were recovered in numbers comparable to those given by the his^- $threo^-$ strain. A comparable increase in one of the other four requirements of the strain, leucine, brought no similar increase in histidine reversion.

Further experiments have shown that in the presence of 1 μg/ml of histidine, addition of leucine, uracil, tryptophan, and threonine each at 30 μg/ml, or singly or in combination at 100 μg/ml, yielded numbers of histidine revertants solely dependent on the threonine concentration. The deprivation of threonine is thus the only discriminatory property practised by the his^- $threo^-$ background.

It still remained possible that the different yields of histidine revertants on the various concentrations of threonine (Table 37) were not caused by the permission of revertant

TABLE 37. Effect on Yield of his^+ Revertants of Varying Concentrations of Threonine or Leucine. - 5 experiments.

CONCENTRATION OF TESTED AMINO ACID IN SELECTIVE MEDIUM (μg/ml)		HIS^+ REVERTANTS PER MEAN OF 10 PLATES OF EXPERIMENT NUMBER:				
Threonine	Leucine	1	2	3	4	5
30	30	1	3	9	4	5
60	30	12	38	39	54	35
100	30	24	35	71	73	77
300	30	166	102	142	54	69
30	60	1	1	0	-	-
30	100	1	2	3	-	-
30	300	1	3	2	-	-

Unless stated to the contrary, selective medium contained 30 μg/ml each of leucine, uracil, tryptophan, and threonine, and 1 μg/ml histidine. Approximately 10^7 cells (a constant number in each experiment) of the his^- $threo^-$ strain was inoculated per plate.

growth by suitable threonine concentrations. If the amount of background growth of the initial inoculum was in some way dependent on the threonine concentration rather than on the apparently limiting 1 μg/ml of histidine in the agar, a similar result might be expected.

Experiments were then designed to test the effect of these various threonine concentrations on background growth. Inocula of his^- $threo^-$ cells were spread on plates of selective agar with 1 μg/ml histidine and either 30 or 100 μg/ml of threonine. At daily intervals, the crop of cells was washed off replicate plates and viable counts performed. In all these experiments, the extent and rate of background growth of the his^- $threo^-$ inoculum was the same whether 30 or 100 μg/ml of threonine were present. In contrast, inocula of his^+ $threo^-$ cells grew to at least 5-10 times the cell density of the his^- $threo^-$ inocula, demonstrating that histidine was the limiting factor for his^- strains. Obviously 30 μg/ml of threonine allows full background growth of the his^- $threo^-$ strain, but not growth of its his^+ revertants which arise late on the plates, while the only limiting factor to this background growth is the 1 μg/ml of histidine. 30 μg/ml of threonine is the quantity normally used to give wild-type growth rate and final cell density to the threonine-requiring single (AP3) and multiple (UV2 + AP3) auxotrophs.

In a final series of experiments, the specificity of discrimination has been examined. Histidine revertants were discriminated against only from a his^- $threo^-$ parent.

The his^- $threo^+$ strain yielded revertants in the presence or absence of gratuitous threonine however. The threo marker reverted spontaneously in a his^- $threo^-$ strain, also in a his^+ $threo^-$ strain in the presence of gratuitous histidine. It will be of interest for the future to test if revertants of other markers arise during residual growth on selective media, and whether they too are discriminated against in the threonineless background.

It is important to point out that the discrimination occurred only for spontaneous histidine revertants arising on selective plates. Yields of EMS-induced supersuppressor revertants of the his-A1 marker and his^+ transformants produced with wild-type DNA were not affected by the threonineless background, even on the agars most suited to discrimination. These revertants and transformants are present however at or shortly after the time of plating (dependent on their respective times of incorporation and expression), i.e. before discrimination becomes effective.

D I S C U S S I O N

D I S C U S S I O N.

At the outset of this work, the main aim was defined as the elaboration of mutational systems in transformable strains of B. subtilis. Reference was to be paid to the effects of ancillary conditions, particularly those of nutrition, on mutation frequencies induced by UV and some chemical mutagens. Such physiological effects have indeed been described, both of general and strain-specific nature:

a. a general phenomenon of the enhancement of mutation yield by traces of the previous growth requirement,

b. a further general phenomenon of an additional enhancement of mutation yield by traces of an amino acid pool,

c. a strain-specific sparing of MFD by some of the previous growth requirements of strain UV2 and its derivatives, particularly by leucine and uracil, and

d. a specific selection against revertants of the his-Al marker in a threonineless background.

It is necessary to precede discussion of these several phenomena by a description of some of the introductory work. Reversion spot-tests had demonstrated that few of the markers borne by strains obtained from other laboratories were likely to be of practical value for mutation studies (Table 4). Indeed, of these markers, only his-Al has been used consistently throughout this work. The uniform size of its revertant colonies, hopefully indicative of the correction of a single type of genetic lesion, and the ease of induction of these revertants with some alkylating agents are the primary attractions of this marker. Two

interesting phenomena described with this marker will be subsequently discussed - suppression, and the selection against spontaneous revertants by a threonineless background already referred to.

The lack of suitable markers for reversion studies led to a programme of induction and classification of new markers. As the organism has been so little used for mutation studies to date, some incidental observations on the spectra of induced mutation may be of interest and will be briefly discussed. It should be recognised, however, that this part of the work only constitutes the "tooling-up" of the species for mutation study. In no way was it intended to be a quantitative study of forward mutation, nor was the elucidation of molecular mechanisms responsible for mutation spectra a prime object of the investigation.

INDUCTION AND TESTING OF NEW AUXOTROPHS.

1. Selective Technique.

Although the auxotrophic markers were selected either with penicillin screening or by thymine starvation, there is little evidence that the method of selection imposes a specificity of recovery of any particularly favoured nutritional types. A possible exception in this regard may be the recovery of a higher proportion of leaky mutants by thymine starvation, although this and other specificities may be a property of the respective mutagens.

During the course of auxotroph recovery, it was found that a single cycle of the selective processes for mutant

enrichment was insufficient to return a good yield. This was particularly evident with UV and 2AP, where 5 and 7 auxotrophs respectively were recovered from approximately 1000 tested colonies with each mutagen. There can be no reason to doubt the mutagenicity of these two agents in B. subtilis, although its degree may leave something to be desired. UV reverts many markers (Tables 4, 22, 23) and 2AP induces reversion of the ura-1 marker (Figure 10).

An equally low yield of auxotrophs was recovered after treatment with a mutagen well known for its great efficiency in other systems, EMS. In this case, a second cycle of penicillin selection was also given, resulting in a great increase in the number of recovered auxotrophs. Thymine starvation was equally inefficient as a selective technique, used in single cycle, although more than compensated for by the great mutagenicity of the agent used in this particular case, NTG. The poor performance of both selective techniques when given in single cycles underlines the desirability of repeating them. It is true that Nester et al. (1963) claimed a recovery of large numbers of auxotrophs after UV and a single cycle of penicillin selection, but this was almost certainly due to the magnitude of the dose applied, giving about 0·01% survival compared to 50% in the present work.

b. The Nature of Newly Induced Auxotrophs.

The newly induced auxotrophs have been classified according to growth requirement, and spontaneous and EMS-induced reversion in spot-tests. The four mutagens used

for forward induction are known to produce predominantly point mutations in other systems: 2AP inducing transitions, UV transitions and frameshifts, EMS transitions and rare deletion, and NTG transitions, also transversions in other systems (Baker and Tessman 1968, Bautz and Freese 1960, Drake 1963, 1964, Krieg 1963, Margolies and Goldberger 1968, Whitfield et al. 1966). The data obtained in the present work do not contradict this general conclusion, and suggest that, for the most part, the EMS-revertible mutants of B. subtilis are point mutated by base pair substitution.

It is appropriate to enter here a word about the role of suppression in reversion spot-tests. No attempt has been made in the present work to distinguish between true and suppressor reversion, other than by scoring large colony revertants growing at rates approximating to wild-type in order to eliminate slow-growing partial or suppressor revertants. It seems likely that the majority of revertible mutants induced in the present work represent base pair substitutions. These mutants were induced with agents known to produce such mutations, although apparently only 2AP and NTG do this to the exclusion of other mutational types in other systems (Whitfield et al. 1966, Oeschger and Stahl 1967, Margolies and Goldberger 1968). The agent used to spot-test them for reversion, EMS, similarly induces predominantly base pair substitutions, although rare base deletion may result from the hydrolytic loss of ethylated guanine (Krieg 1963). Base pair substitutions may produce nonsense or missense, each of which may be truly or partially

reverted, or externally suppressed (e.g. Benzer and Champe 1962, Garen and Siddiqi 1962, Epstein et al. 1963, Notani et al. 1965, Reid and Berg 1968, Regos and Szende 1968). In contrast, only one instance is known to the present author where a frameshift, the second class of point mutation, is suppressible by an external suppressor. This is an extraordinary, and surely rare, event in S. typhimurium where the suppressor is believed to assist reinitiation of in phase translation after its disturbance by a frameshift-generated nonsense codon (Riyasaty and Atkins 1968). At the level of quantitation on which this preliminary investigation was performed, further genetic analysis of revertants was deemed unnecessary.

c. Comparison between Forward Mutagens.

The auxotrophs induced in B. subtilis are classified in Table 38 according to their pattern of reversion and their leakiness. A number of specificities are apparent:

1. the proportion of forward mutants displaying leakiness was much greater for 2AP and NTG induction than for EMS induction. It is not known whether these leaky mutants still have residual activity of a mutated enzyme, or whether they are bradytrophs, where a leaky phenotype remains despite the total abolition of an enzyme activity.

2. a greater proportion of mutants induced by NTG were stable to reversion than of those induced by EMS. It is evident that mutants which revert spontaneously may well revert with a number of mutagens dependent on chemical and other specificities, although obviously not with EMS in the

TABLE 38. Classification of Newly Induced Auxotrophs of B. Subtilis according to their pattern of reversion.

REVERSION CLASS OF AUXOTROPH.	SPONTANEOUS REVERSION.	EMS-INDUCED REVERSION	AUXOTROPHS INDUCED WITH				TOTAL NUMBER.
			UV	2AP	EMS	NTG	
A	-	-	0	0	6	16	22
B	-	+	0	0	12	13	25
C	+	-	1	0	7	3	11
D	+	+	4	7	67	22	100
		TOTALS:	5	7	92	54	158
		NO. OF LEAKY MUTANTS IN TOTAL:	0	2	6	11	19

present circumstances.

The most interesting mutants are those induced by EMS and NTG which are totally stable to reversion under the conditions of test. Such reversion stability may be due to any of a number of likely causes, although only the first two suggestions will apply to revertible mutants with undetectable rates of spontaneous reversion:

a. choice of an unsuitable agent (in this case EMS) to revert a base pair substitution, which should however respond to other mutagens of different chemical capability.

b. lack of response of a frameshift mutation to EMS. Although EMS is reported to induce rare base pair deletions in phage T4 (Krieg 1963), there is no evidence of frameshift mutation induced by NTG in S. typhimurium (Whitfield et al. 1966, Oeschger and Stahl 1967, Margolies and Goldberger 1968).

c. multisite mutation in a number of genes, as reported for NTG in S. typhimurium (Loper et al. 1964, Eisenstark et al. 1965) and E. coli (Adelberg et al. 1965, Hirota et al. 1965), and for EMS in S. cerevisiae (Lindegren, Courtis, and Shult 1968).

d. multisite mutation in single genes, i.e. a linear array of adjacent yet recombinationally separable genetic sites, or chromosomal aberrations. Such mutants in the histidine operon of S. typhimurium have been found never to revert either spontaneously or on induction, although they are transducible to prototrophy with phage prepared in a wild-type

strain (Loper et al. 1964). Reversion-stable ad-3B mutants of N. crassa induced with EMS have been suggested to carry such multiple mutations (Malling and de Serres 1968). Intragenic deletions may also be induced in N. crassa with EMS (Malling and de Serres 1968), although definite proof is lacking.

Without further genetical studies, it is not possible to state unambiguously that one particular mechanism is responsible for reversion stability of any of the relevant mutants of B. subtilis. Examination of Tables 7 and 8 does suggest, however, that multisite mutation in a number of genes is not the cause, for 20 of the 22 mutants in this class had either a single growth requirement or a number of requirements compatible with mutation in single genes (i.e. ilva, ser+gly, am, aro, cys+met). The remaining two mutants, of complex phenotype, may bear such multisite mutation in a number of genes.

There is no evidence for specificities of mutation induction at the species level, as shown by a comparison of the new auxotrophs of B. subtilis with those induced in other organisms by the same or comparable agents.
2 differences are evident, however, between B. subtilis and S. typhimurium, although both are probably attributable to technique:

1. comparison of the present work with that of Eisenstark et al. (1965) reveals a difference in spontaneous revertibility. Of auxotrophs both induced and reverted by ethylating agents, the majority of those of B. subtilis were also

spontaneously revertible. In contrast, the majority of S. typhimurium auxotrophs in this class were stable to spontaneous reversion. This may possibly indicate a true difference in the mutation spectra induced by ethylating agents in the two species. A more probable explanation lies in the sensitivity of the test for spontaneous reversion in the two systems.

In both cases, spontaneous reversion was detected following the plating of an inoculum of the studied strain on trace supplemented agar. The final population size attained on the plate will depend on the amount of residual division afforded the inoculum, and this in turn will depend on the amount of trace supplement in the selective medium. The number of spontaneous revertants observed will be proportional to the final population size, unless interfered with by genotype or phenotype. Thus we may expect to detect rare spontaneous reversion with regularity only with the large populations achieved with high levels of trace supplementation. A technical comparison of the two systems shows that greater quantities of richer "Oxoid" nutrient broth were used for supplementation in the present work than were used of the poorer "Difco" nutrient broth by Eisenstark et al. (1965). The test conditions for B. subtilis thus permit more residual division and a greater sensitivity of the test. Mutants with infrequent spontaneous reversion in B. subtilis may therefore be classed as "positive", although they might appear "negative" with reduced supplementation.

2. NTG-induced auxotrophs have been recovered both from B. subtilis in the present work and from S. typhimurium by Eisenstark et al. (1965). Some auxotrophs of both species were stable to reversion, both spontaneous and induced with ethylating agents. 16 such mutants were recovered from B. subtilis, 14 of which had either single growth requirements or multiple requirements compatible with mutation in single genes (i.e. am, aro, ilva, cys+met). The other two mutants were of complex phenotype, suggesting possible multiple mutation in more than one gene.

In contrast, however, 8/15 reversion-stable auxotrophs of S. typhimurium contained multiple mutations in unlinked genes, as deduced from transduction analyses. This difference is also probably attributable to technique. NTG treatment was deliberately curtailed in B. subtilis in order to avoid the induction of just these multiple mutations, and with evident success. In S. typhimurium on the other hand, some auxotrophs were recovered after actively growing cells on agar in the presence of NTG - a procedure likely to result in multiple mutation.

d. Reversion by Combined Mutagen Treatments.

One of the potentially interesting markers available for study is ura-1. A comparison of induced reversion of this marker and the his-A1 marker reveals a mutagen specificity (Table 4). Ura-1 reverts with UV and with 2AP, whereas his-A1 responds only to UV.

Such mutagen specificities may be perfectly well explained

in a facile manner at the level of the primary mutagen: DNA interaction. That is to say, the genetic lesion necessary to revert ura-1 may be brought about both by 2AP and UV, whereas the different lesion necessary to revert his-A1 may be brought about only by UV. In B. subtilis, for example, 2AP may cause transitions, predominantly AT to GC, whilst UV may cause transitions, predominantly GC to AT, and frameshifts. Use of such chemical specificities with carefully defined systems has led to some of the great advances of molecular genetics.

It should not be forgotten, however, that in addition to the production of lesions in DNA in unknown frequencies, mutagens may also affect other cellular processes, and structures. In the present case, for example, with this in mind, we could reinterpret the data as follows: lesions induced both by 2AP and UV cause the induction of reversion of both markers, ura-1 and his-A1. Whereas ura-1 revertants induced by either agent give rise to mutant colonies, only his-A1 revertants induced by UV do so. The his-A1 revertants induced by 2 AP on the other hand may be prevented from growth by some action of the 2AP on the pathway to expression of histidine revertants. Interaction experiments were therefore performed to test whether such 2AP treatment in any way inhibited the subsequent reversion by UV of the his-A1 marker. The results were negative (Table 30).

Such an interaction has been shown in Neurospora, however (Auerbach and Kølmark 1960, Auerbach 1965 and in press). In a doubly auxotrophic strain, DEB pretreatment

was found to enhance adenine reversion frequencies induced either by further DEB treatment or with UV. Such pre-treatment simultaneously decreased the reversion frequencies of the second marker, inos$^-$, by such subsequent doses of DEB or UV. A preliminary DEB treatment therefore appears to enhance the realisation of adenine revertants whilst simultaneously decreasing that of inositol revertants, not necessarily induced with the same agent. The clue to this interaction effect had come from the differential responses of the two markers to DEB, in analogous manner to the effects of 2AP on the two markers of B. subtilis. Unfortunately the situation in B. subtilis did not prove so rewarding as that of Neurospora.

As DEB and UV had thus been proved to show the enhancement of mutation frequencies by action at the cellular level in Neurospora, this combination was also tried in B. subtilis with the his-Al marker. This marker responds weakly to both mutagens, and the interaction experiments were performed to test whether DEB pretreatment affected the subsequent UV-reversion of the marker. (Table 29). In this instance also, a negative result was recorded. It will be instructive to search for more sites of possible interaction, in B. subtilis, be it synergism, or antagonism, where the property of transformation should allow analysis of such phenomena by separation of DNA and cytoplasmic effects.

It should be borne in mind also that UV doses were always limited in the experiments reported with B. subtilis for fear of inducing defective prophages. These experiments

will therefore be repeated when this complicating lysogen is rendered uninducible. The discovery of a mutant bacterial strain uninducible for the phage, with readily transformable property of uninducibility, makes this an imminent event. (Karamata 1968).

QUANTITATIVE INDUCTION OF REVERSION.

a. Nature of the Revertants.

Induced back-mutants may be reverted at the site of the original mutation, or by intra- or extra-genic suppression. In only one case in the present work can revertants be unambiguously placed in one of these categories. Reversion to joint independence of histidine and leucine constitutes supersuppression, as has been genetically demonstrated (Tables 13, 14, and the text). All other revertants of other markers may yet be of any type. Some genetical studies have been undertaken with revertants of some markers with a view to such classification. Reisolation of original auxotrophies was attempted by crossing revertants to wild-type (DNA), but this procedure proved both laborious and unrewarding.

Of the remaining markers, therefore, all that can be said is that any revertants show or do not show wild-type growth rate, and thus may or may not be true revertants. By this means we may say that backmutation of the ura-1 marker is definitely not to wild-type as its revertant colonies show very slow growth. Similarly, as revertant colonies of his-B2 are of more than one colony size, multiple

are the means of its reversion.

Ability to distinguish between revertant types would be very much facilitated by the development of phage mutant systems. Use of nonsense mutants of phage T4, for example, has allowed the speedy classification of large numbers of revertants of a few bacterial markers (Osborn and Person 1967). These test systems may be enlarged in the future by the inclusion of certain missense mutants of the phage capable of being supersuppressed, and by the use of suitable mutations borne on episomes. B. subtilis is possessed of many phages, and such mutant systems may soon be developed.

Unfortunately, there is no evidence to date for true reversion in any mutant of B. subtilis. This is particularly regrettable due to the claim, for E. coli B/r WP2 try$^-$, that UV-induced suppressor revertants display a sensitivity to ancillary conditions not shown by true revertants of the same marker induced simultaneously. It is essential that this reputed specificity for suppressors of the broth effect and MFD be confirmed or denied both in the system of its original description, and, if possible, for other mutagens and other organisms.

b. Mechanism of Supersuppression.

Demonstration of supersuppression for revertants of the his-A1 and leu markers of strain UV2 immediately excludes mechanisms of suppression active only upon single specific alleles (e.g. Brody and Yanofsky 1963, Barnett et al. 1967), or acting only on functionally related loci of one particular pathway (e.g. Kakar 1963). The B. subtilis mutants are

certainly allele specific suppressors, without locus specificity, as are the supersuppressors of yeast (Hawthorne and Mortimer 1963, Mortimer and Gilmore 1968). These yeast supersuppressors are believed to act in analogous manner to some suppressors of E. coli (Epstein et al. 1963, Brenner and Beckwith 1965, Zipser 1967) by altering the fidelity of translation. Although no studies have yet been made to elucidate their mechanism of action, it is probable that supersuppressors of B. subtilis also act at this level.

In phage T4, mutations responding to this type of suppression have been shown to code for nonsense (Brenner et al. 1964) terminating the translation of messenger RNA (Sarabhai et al. 1964), and also for certain missense (Reid and Berg 1968). The mechanism of suppression of both these types of mutation is believed to involve mutationally altered tRNA's capable of inserting suppressor-characteristic amino acids at sites corresponding to the mutated codons (Capecchi and Gussin 1965, Engelhardt et al. 1965, Carbon, Berg, and Yanofsky 1966, Gupta and Khorana 1966, Gesteland et al. 1967).

It is possible that this suppression by altered tRNA may act only when genetic redundancy exists for the particular species of tRNA. This would allow production of the necessary normal tRNA by the unmutated genes and of the su^+ tRNA by the mutated gene (Landy et al. 1967). Suppressors of this type, however, may in some cases have additional pleiotropic effects, for example, upon the ribosomes. A recent suggestion made to explain this pleiotropy is that

some suppressor mutations arise in the structural gene not of a tRNA but of a modifying enzyme. Modification of nucleic acids is a well-known phenomenon, arising by methylation, glucosylation, or pseudouridylation, and the postulated enzyme could well alter the coding specificity of a tRNA by modification of a base at the anticodon site. In this case, it would be the enzyme that was mutated to a different substrate affinity, and this could well affect other such cellular components as the ribosomes, etc., to produce the pleiotropic effects (Gartner et al. 1969).

c. The Enhancement of Mutation Yields by Nutrients, and MFD.

Two general means have been found of enhancing recovered mutation frequencies. Firstly, by adding to the selective agars traces of the previous growth requirements. This was detected both for EMS and NTG, although the data are too small to allow its definition for UV and DEB due to the weak mutagenicity of these agents in this system. The data for MMS are too variable for inclusion in this discussion. For both EMS and NTG, the effect shows a similar dependence of magnitude on dose. Secondly, an additional effect of enhancement by an amino acid pool, usually supplied as broth or casein hydrolysate plus tryptophan. This effect was detected with all mutagens used, although in contrast to the specific requirement effect, its magnitude was dependent not only upon the dose but on the mutagen.

1. The Specific Requirement Effect.

The stimulation of mutation yield by traces of previous

growth requirement alone may reflect a need for residual synthesis and division. There are a number of ways in which this need may arise, although it is not possible to say which pertains to B. subtilis:

a. acting at the level of the mutagenic agent - many mutagens, whether causing DNA damage or acting as base analogues, actually induce a mutagenic event during replication of the genetic material following their application to cells or their incorporation into DNA. Alkylating agents induce such errors of replication, as do the base analogues 2AP or 5BU, which may also induce errors of incorporation. A treated cell may thus be potentially mutant although not becoming functionally so without one or more DNA replications. Margolin and Mukai (1961, 1964) have claimed that the division dependence apparent in the reversion of some strains of S. typhimurium is caused by such a phenomenon. Leucine auxotrophs were classified according to 2AP-induced reversion into two types - the first giving a large mutant yield without such residual division (division-independent), the second giving only low mutant yields without division. although greatly enlarged yields when permitted division (division-dependent). Postulated explanations were that the two types of mutation either depended on the type of transitions induced by 2AP being different in the two classes, or whether a particular transition was induced on the coding strand of DNA or its complement. The evidence for either explanation cannot be said to be convincing, although there is no reason to doubt the general application of errors of

replication and incorporation. In the present example, however, need for expression may be the basis of division into two classes.

b. induced mutants are selected from a majority of unmutated cells by imposing a suitable challenge. Fitness to overcome this challenge may depend on a mutant cellular product, be it enzyme or structure, which must obviously be manufactured in sufficient quantity before the challenge becomes absolute. For auxotrophs, enzyme may have to be made, for streptomycin-resistant mutants a new population of antibiotic-resistant ribosomes, etc. Without such synthesis, a cell will be mutant genotypically, but not phenotypically, and may therefore be killed by the selective challenge.

It has recently been shown in a system where suppression is phenotypic due to antibiotics that a small amount of "primer" growth requirement is required before suppression becomes effective (Kirschmann and Davis 1969). Unless completely blocked auxotrophs were given enough growth requirement for up to one division, suppression was not manifested by the presence of streptomycin or chloramphenicol. The efficiency of this antibiotic-induced suppression thus appears to be so low that insufficient active enzyme is made on selective media unless residual synthesis is permitted. When a sufficiency of enzyme is achieved, growth in the presence of the antibiotic should continue in the absence of the amino acid.

Such phenotypic suppression is well-known to occur by alteration of the fidelity of translation by the antibiotics. Interference with translational fidelity is also the end

result of the differently based genotypic suppression by altered tRNA. The induction of suppressor reversions may thus be subject to a similar need for residual synthesis if suppression is weak,

c. in addition to the possible strand separation mentioned in a., two other classes of segregation effect may be expected in certain situations. Firstly, where the newly mutant allele is recessive, nuclear segregation will be necessary in the presence of its dominant allele where the cells are multinucleate. Secondly, where one of a number of redundant genes is mutated, the mutant gene product may have to compete with the products of nonmutant genes. This competition may be unfairly altered by a multinucleate condition of cells, where the ratio of nonmutant:mutant alleles will be further increased to the detriment of mutant function. Nuclear segregation may return a favourable balance and allow growth of the mutant.

All three possible mechanisms are dependent on the permission of growth, either residual synthesis or division, for a short time on complete medium or by the trace supplementation of selective media. For reversion to prototrophy at least, one may speculate that a stimulatory effect of the previous growth requirement will not be found under two conditions - where the initial auxotroph is leaky, or where intracellular pools of requirement are high. Kirschmann and Davis (1969) have already shown that leaky mutants do not require a "primer" of previous growth requirement for the establishment of phenotypic suppression by antibiotics,

presumably because they can supply their own.

The dose dependence of the previous growth requirement effect is highest at the lowest mutagen doses with EMS and NTG, reducing thereafter. This may be due to a greater turnover of the trace supplement in the medium during the increased delay caused to treated cells at the bigger doses. Otherwise, the dose dependence may reflect a further general effect on the induction of su^+ mutants which is interfered with to greater or lesser extent by different doses of mutagen, or an effect specific to each mutagen and dose dependent. The similar dose dependence of the effects for EMS and NTG suggests that the effect is general in nature. The two mutagens do differ inexplicably, however, in that MFD is shown of mutants recoverable on agar supplemented with traces of previous growth requirement when induced with EMS, but not with NTG. (Figures 3 and 9).

2. The Broth Effect and MFD.

Addition to the selective agar of broth allows two simultaneous effects - a stimulation as just described due to the broth's content of previous growth requirements, and an additional effect due to an amino acid pool. This extra effect of broth was detected for all mutagens used with B. subtilis, whether UV or alkylating agent, with the exception of 2AP for which it was not sought. In contrast to the effect of previous growth requirement, the broth effect varied not only by dose but by mutagen. For EMS, UV, and DEB, the broth effect was smallest at low doses, increasing

thereafter to relatively constant values. The broth effect for NTG on the other hand was greatest at low doses, reducing thereafter, in the manner of the previous requirement effect. It seems likely that the broth effects described for B. subtilis represent a general phenomenon, which may be variously affected by different doses of different mutagens. This is in contrast to E. coli, where the broth effect shown by strain B/r WP2 for tryptophan reversion appears to be a characteristic of the mutagens, occurring after UV, but not after ionizing radiation, NMU, or DEB (Bridges et al. 1968, Clarke 1969).

The broth effects described in B. subtilis for EMS and in E. coli and S. typhimurium for UV demonstrate a number of points in common:

i. they are due to a pool of amino acids other than those specifically required by the strain,

ii. broth must be present from the time of mutagen treatment to a time approximating to that of the first post-treatment division,

iii. broth acts on solid medium as well as in liquid medium provided that a suitable broth:cell ratio is maintained,

iv. in an absence or insufficiency of broth, an irreversible decline in mutation frequency occurs (MFD) of those mutants recoverable on broth-supplemented agar,

v. MFD is temperature-dependent and may be prevented by holding treated cells in the cold,

vi. the broth effect after UV with S. typhimurium increases with the richness of the previous growth medium. This is due

to a depression of mutation yield on broth-free agar rather than an increase in overall mutability as measured on broth-supplemented agar. The broth effect after EMS in B. subtilis shows the same relationship to the previous growth medium. It is unwise to generalise about this effect of previous growth medium, however, as E. coli displays great variability in this respect (see discussion in Clarke 1969).

Of particular interest, the broth effect and MFD have been claimed to be suppressor-specific after UV in E. coli, at least for strain WP2 (Bridges et al. 1967). The effects certainly occur for EMS-induced suppressor reversion in B. subtilis, but demonstration of suppressor-specificity must await the availability of truly revertible markers.

In the E. coli systems, the majority of UV-induced lesions are presumed to be repaired in resistant strains. The unrepaired residue gives rise to all or a substantial part of induced mutation, although the limited participation of irreparable lesions cannot be excluded. In the absence of postirradiation protein synthesis, however, an additional fraction of mutation-inducing lesions is repaired, this being detected as MFD (Witkin 1966). This extra MFD-repair has a number of properties in common with pyrimidine dimer excision, both requiring a carbon source, being blocked by acriflavine, and being absent from the UV-sensitive derivative $WP2_s$ hcr^-. Mutants isolated as unable to perform MFD (mfd^-) have been shown to have a reduced rate of dimer excision (Setlow J.K., quoted in Witkin 1966). The two processes differ, however,

in their relative efficiency at different stages of culture, and in their response to certain posttreatments. MFD probably represents the removal of some pyrimidine dimers that can only be efficiently excised when the overall efficiency of dimer excision is low (Witkin 1966), i.e. it constitutes an exceptional rather than a normal mode of repair.

Recent advances in the control of macromolecular synthesis in E. coli allow a hypothesis to be made to explain the broth effect of inhibition of MFD. Strains of E. coli stringent for control of RNA synthesis (RC^{str}) may be starved of amino acids in two ways - either by the removal of a required amino acid, or by a step-down from rich to poor medium. The parallelism is apparent between these conditions and the plating of treated cells on a minimal medium to select revertants to prototrophy. Under amino acid starvation, the synthesis of transfer and ribosomal RNA is immediately repressed, although the synthesis of some messenger RNA may continue, particularly of the tryptophan operon (review - Edlin and Broda 1968). When amino acids are present, however, as when plating on broth-supplemented agar, synthesis of transfer and ribosomal RNA continues along with that of some messenger RNA. The difference between the two conditions therefore is that the genes for tRNA, where some suppressor loci are situated, are repressed or derepressed dependent on the presence of an amino acid pool. The state of structural genes for enzymes whose mutation is under study will not be expected to vary greatly

in the presence of traces of its end-product supplied alone or in broth.

It is known that genes vary in their susceptibility to repair according to their states of repression or de-repression. Kölsch and Starlinger (1965) have shown that UV-inactivated galactokinase activity of E. coli is photo-reactivated when repressed, although no such photorepair can be detected in the presence of inducer. It is possible that this variable sensitivity to photorepair may also apply to the other cellular repair mechanisms, these too requiring access to the DNA in order to repair it. If this is true, then under the step-down conditions when MFD occurs, suppressor mutations situated in tRNA genes may be preferentially repaired, as has indeed been claimed by Bridges et al. (1967).

This hypothesis raises an interesting speculation. In the relaxed mutants of E. coli (RC^{rel}), such effects of step-down are much less severe, and the synthesis of transfer and ribosomal RNA continues under conditions of amino acid deprivation. It might therefore be expected that in these relaxed strains MFD should not be suppressor-specific. As the conditions of amino acid starvation do not constitute a step-down for these mutants, their tRNA genes should remain derepressed, and they should now have similar sensitivity to repair to the structural genes.

The effects described in B. subtilis may be attributable to similar mechanisms. Repair processes, such as appear to be involved in MFDin E. coli, demonstrate great catholicity of function (e.g. Hanawalt and Haynes 1965, Kohn, Steigbigel,

and Spears 1965, Strauss et al. 1966), and are thus capable of acting on potentially mutagenic lesions induced by both UV and alkylating agents, the mutagens used in the present work. Other explanations of a more trivial nature seem less probable: indeed, in so far as was possible, mutagen-induced osmotic instability, involvement of an agar component in conjunction with broth, an action of residual mutagen which is washed out of cells during posttreatment, and liquid holding recovery have been excluded. These last two were of particular importance due to their respective precedents in Neurospora and Ophiostoma (Kølmark and Kilbey 1962, Zetterberg 1966).

There are two differences outstanding between the broth effects and MFD described for B. subtilis and E. coli:

i. MFD in strain UV2 of B. subtilis and its derivatives after EMS treatment was greatly reduced by addition to liquid medium of some of the specific growth requirements, among which leucine and uracil must be numbered. No such effects have been reported for E. coli. This is likely to be a strain rather than a species specificity, however, as a number of the new auxotrophs of B. subtilis showed it only in the strain UV2 background, never as monoauxotrophs.

ii. no broth effect was found for mutagenesis by NMU or DEB in E. coli (Clarke 1969), although the present work describes broth effects in B. subtilis with four alkylating agents, including DEB. The reported absence of MFD after NTG treatment of B. subtilis by Yoshida and Yuki (1968) is almost certainly attributable to the use of broth-free plating

medium upon which such effects would not be detected.

As a control for medial effects on the induction of mutation in B. subtilis, such effects were also sought after the introduction of new characters to the cells by genetic transfer. These studies were not pursued far in their own right, but they do reveal several interesting features worthy of independent investigation.

A broth effect was detected for the transformation of all markers tested, whether single or double wild-type, or suppressor, although no effect of the previous growth requirement was evident. Although one might conclude that broth effects occur whenever a new marker is introduced into a cell, regardless of the means of its introduction, it is likely that the broth effects for transformation constitute a general enhancement of recombination and expression, following the uptake of incoming fragments of donor DNA. It should perhaps be repeated here that this broth effect is definitely not upon DNA uptake, as this stage of the reaction is terminated with DNAase before the cells are ever exposed to broth. A similar enhancement of transformant yields with an intermediate incubation on complete medium has been reported by Jensen and Haas (1962). Such enhancements by nutrient may be explicable as follows: cells competent for transformation are in a state of arrested DNA synthesis, the stationary replicating points varying in their chromosomal location from cell to cell (Bodmer 1965). Integration of transforming DNA appears to be related in some way to these

sites of replication (Bodmer 1966), although it does not necessarily occur at these sites (Laird, Wang, and Bodmer 1968). The base line for transformation will therefore be drawn by the relationship between the incoming fragments of donor cell DNA and the availability for recombination of their specific chromosomal equivalents near replicating points. The time-dependent shifts in transformation frequency of different markers by a uniform preparation of DNA in synchronised recipient cell cultures is consistent with this hypothesis (Erickson and Braun 1968). If the donor DNA fragment is not chromosomally integrated, it is degraded. In the presence of broth, however, chromosomal synthesis may recommence earlier, with its renewed movements of the points of replication. This may present extra opportunity for unintegrated donor DNA fragments to locate their chromosomal equivalents near a replicating point before they undergo degradation. Hence the apparent stimulation of transformant yield by broth. Integration of one DNA fragment may also induce a new point of replication at the site of this first integration (Laird et al. 1968). The co-transformation of linked markers is explained in this hypothesis by the uptake of more than one donor DNA fragment per cell, and the well-known existence of more than one replicating point in individual cells.

Medial effects on transduction on the other hand varied not only by experiment, but for different markers in each experiment. It is likely that a great deal of this variation may be explained by the concurrent phage infection. One

interesting and repeatable observation stands out of these experiments, however. In contrast to suppressor mutants and transformants, no such transductants were ever recovered on unsupplemented selective agar (Table 33). The stimulation of transductant yield of suppressors by previous growth requirement is obviously specific to these suppressors. It is probable that this effect as well may be reflecting metabolic differences imposed by the concurrent phage infection.

THE ANALYSIS OF AN APPARENT CASE OF "GENE-CONTROLLED MUTATIONAL STABILITY".

The his-A1 marker of strain UV2 spontaneously yields revertants which are predominantly his^+ leu^-, whilst on induction with a variety of mutagens $su^+_{his\text{-}A1}$ leu mutants are recovered. The his^+ leu^- mutants do not unfortunately appear to be inducible with any agent used so far. Very few spontaneous his^+ leu^- revertants are recovered from this strain on plating on unsupplemented selective agar, but addition of traces of histidine to this agar yields many revertants, with an independence of number on plating density (Table 10). The simple explanation for this lack of dependence on inoculum size appears to be that almost regardless of the number of cells originally inoculated per plate, a given amount of trace supplement permits growth to a characteristic population density. As the number of histidine revertants will ultimately depend upon this relatively constant final population size achieved on the plates, rather than on the size of the original inoculum, their number also appears

to be relatively independent and constant over a wide range of initial plating densities (Table 10). For example, from a his^- $threo^+$ inoculum of strain UV2, between 50 and 200 revertant colonies will be recovered with a trace supplement of 1 μg/ml of histidine or 1% v/v. nutrient broth, dependent on the experiment. This test for spontaneous reversion is quite sensitive, as populations of over 5×10^8 cells may be achieved per plate. It also allows the easy detection of any effects on reversion by genetic background or medial factors.

Using this technique, effects of genetic background were sought on the spontaneous and induced reversion of the his-Al marker (Table 11). No effects were apparent on the induction of reversion to $su^+_{his\text{-}Al}$ leu, but an inhibitory effect of a $threo^-$ marker on spontaneous reversion was detected. When inocula of a his^- $threo^-$ derivative of strain UV2 were plated on the relevant assay media for histidine reversion with trace supplements, very few revertants were recovered. In the first few experiments, in fact, with a trace supplement of 1 μg/ml of histidine, not one histidine revertant colony was found. Some factor associated with the threonine requirement apparently stabilises the his-Al marker to spontaneous reversion. $Threo^+$ derivatives of the $threo^-$ strain showed normal histidine reversion.

The amino acid, threonine, has itself been excluded from responsibility for the inhibition (Table 34). Other growth substances have been previously implicated in two types of inhibition of mutant cell growth in other systems.

Firstly, by an indirect effect, a background of nonmutated cells may either so deplete the medial energy sources that mutant cells are unable to grow (Ryan and Schneider 1949, Grigg 1958, Howe and Dawson 1968) or produce substances inhibitory to mutant growth (Ryan and Schneider 1949). The his-A1 marker reverts freely in a threonine-independent background, however, and it seems improbable that addition of a single threonine requirement so deranges cellular physiology that this kind of indirect inhibitory effect occurs. Secondly, and already excluded for B. subtilis, mutant cell growth may be inhibited by a medial component, be it gratuitously added or required by the studied strain.

An example of this type of inhibition comes from S. pombe (Clarke 1962, 1969) where methionine inhibits the growth of adenine revertants in a manner which depends on the mutagen. The stage at which methionine acts in this example is prior to the formation of completely mutant cells. Other such influences of plating medium are found with Ophiostoma, where it is apparently the common thing for auxotrophs to grow only on a minimal medium with their specific requirement, and not upon complete medium (Fries 1950, Zetterberg 1961, 1962, 1969). In B. subtilis, however, the amino acid is not the inhibitory agent, and the effect must therefore be attributable at some level to the $threo^-$ mutation.

At this stage of the investigation, this inhibition of histidine reversion in a threonineless background appeared to be strikingly reminiscent of the phenomenon of

"gene-controlled mutational stability" reported in E. coli B/r by Chopra (1967), already referred to in this thesis. To recapitulate briefly, the try⁻ marker of strain WP2 attained absolute stability to reversion on addition of a particular adenine requirement to the strain. It was previously reverted both spontaneously and with mutagens, and crossing out of the adenine marker restored its revertibility once more. In this case also, the supplement of adenine was excused responsibility. Other presumed genetic background effects have been reported for E. coli (Glover 1956), Aspergillus nidulans (Morpurgo and Calvori 1966), and S. pombe (Clarke and Loprieno 1965), although effects of the medium have not been rigorously excluded in all these instances.

The situation in B. subtilis does differ from that in E. coli in one crucial manner. Whereas the pathway to adenine reversion in E. coli appears to be completely blocked by an unknown mechanism, small numbers of histidine revertants were recovered in some experiments, particularly when the trace supplement of 1 μg/ml histidine was replaced with 1·0% v/v nutrient broth. Variable numbers of revertants on parallel plates were recorded, as they had been by Grigg (1958) in his study of selection against revertants by the exhaustion of medial energy source. Indeed, such variation in mutant number may be diagnostic for selection against revertants. It is evident that selection against revertants in the present case is unlikely to be at the level of the exhaustion of medial energy source as the fount of inhibition

has been shown to be the threo$^-$ mutation. If a selection against histidine revertants operates in this his$^-$ threo$^-$ derivative of strain UV2, rather than some other type of inhibition, then it must be mediated through this threo$^-$ mutation.

The possible involvement of the threo$^-$ mutation in a selection against histidine prototrophs was examined in two ways. Firstly, reconstruction experiments were performed in which known numbers of either his$^+$ threo$^+$ or his$^+$ threo$^-$ cells were plated out on a background of his$^-$ threo$^-$ cells which had been incubated for 0, 1, 2, or 3 days. These experiments were quite conclusive (Table 36 and text), and showed that fully formed histidine revertants were selected against only by a threonineless background and only if they too were threonine-requiring. Also, discrimination was only seen when revertants were plated later than the background. This suggested an exhaustion of medial threonine by the background. Secondly, when the content of medial threonine was substantially raised, his$^+$ threo$^-$ revertants were formed by the his$^-$ threo$^-$ strain in analogous manner to histidine reversion by the parent strain UV2. (Table 37). This constitutes conclusive proof that the discrimination against revertants by the his$^-$ threo$^-$ strain is due to its exhaustion of medial threonine. An effect of medial threonine on the amount of background growth, with subsequent effect on the numbers of histidine revertants, was previously excluded.

Although discrimination against revertants is known to

be caused by some genetic backgrounds or medial factors, this is the first report of an interaction between genetic background and medial factors. Threonine was the only medial component to be preempted by the auxotrophic background, which obviously leaves a medial sufficiency of leucine, uracil, and tryptophan for the revertants. Examination of the specificity of discrimination showed that there was no evidence of a reciprocal selection against threonine revertants by the histidine marker. Discrimination is only effective against histidine revertants arising late on the plates, and not against those present at the time of plating, induced su^+ revertants or his^+ transformants, both of which last may be considered to arise at or shortly after the time of plating, and before discrimination becomes effective. As a speculation, therefore, one might expect to find that revertants of any other marker which arise late on the plates will be discriminated against in this threonineless background, and it is hoped to investigate this in the future.

In what manner may this preemption of specific growth requirement by an auxotrophic background affect quantitative mutation experiments? Obviously the mutants discriminated against by such a preemption will be those which arise late on the plates. These may be of a number of different types:

1. spontaneous mutants which arise only on plates during residual division (loc. cit., Deering 1963, Chopra 1967),
2. induced mutants arising by a delayed action of mutagenic treatments (e.g. Ronen 1963),

3. mutants induced by massive doses of mutagen which cause simultaneously great delay to mutant growth and division (Ryan 1954), and

4. suppressor or partial revertants with much reduced growth rate compared to their parent wild-types or auxotrophs.

The mechanism of discrimination in the present case is obviously by exhaustion of medial threonine. Some special activity of the threo$^-$ mutation must be responsible, as the other growth factors are not equally exhausted. A likely explanation is that the mutant has an increased rate of threonine turnover due to extra threonine deaminase or dehydratase activity. This may either cause or result from mutation in a gene of the threonine pathway. There are two possible causes for such an increased deaminase activity - either that the mutation in a threonine gene slips the deaminase from regulatory control, or that the mutation causing threo$^-$ phenotype is actually in the threonine deaminase gene, resulting in hyperactivity. Such a situation is known in S. cerevisiae (Brunner et al. 1969), and B. subtilis is known to produce two likely candidate deaminases (Vapnek and Greer 1969). As a further speculation therefore, such discriminatory phenomena may be common when two events interact - a marker whose revertants arise late on plates of trace supplemented agar, and an additional auxotrophy either resulting in or due to the increased activity of a degradative enzyme.

GENERAL SUITABILITY OF B. SUBTILIS FOR MUTATION STUDIES.

The suitability of B. subtilis for mutation studies is illustrated by the similarities existing between it and the strains of E. coli more commonly used:

1. a wide range of mutants may be isolated, with selective properties useful for the performance of mutation experiments. These include both resistances to antibiotics, and auxotrophies for one or more amino acids, bases, and vitamins. Many of these mutants show spontaneous and induced reversion, due in some cases to suppressor mutations.

2. mutagens to which response is shown include UV and chemicals, both base analogue and alkylating agent. There are hints of specificities in the response of particular alleles to the individual mutagens.

3. ancillary effects of nutrition influence the observed mutation frequencies, as may be seen with the broth effect and MFD.

4. other ancillary effects have been shown to be mediated through the genetic background and/or specific medial additives,

5. mutants of increased sensitivity to mutagens have been isolated (Howard-Flanders and Boyce 1966, Strauss et al. 1966). These mutants are apparently unable to efficiently repair genetic damage induced by the respective mutagens.

There is thus considerable common ground between the two systems. In order that the work performed to date should not seem just the direct repetition of that already performed with E. coli, the special properties of B. subtilis should be pointed out, beginning, however, with the

disadvantages:

1. in common with a large number of other bacterial species, including some strains of E. coli, B. subtilis is possessed of defective phage lysogens. These lysogens are known to be induced by some mutagenic agents, and the ensuing cell death provides a possible source of complication for mutation experiments. This nuisance has been avoided in the present work by imposing two limitations on the experimental conditions - by growing the cells prior to mutagen treatment in minimally based media, and by using doses resulting in very little lethality of any mutagen likely to cause phage induction (e.g. UV). Although such limitations are not too serious for preliminary work of the kind reported here, the subsequent development of the system might be much impeded by them. The recent isolation of a mutant strain whose property of uninducibility of these defective lysogens is apparently readily transformed (Karamata 1968) will abolish this disadvantage in the future. The property of uninducibility will be crossed into all strains used in subsequent mutation experiments.

2. to date, no markers of B. subtilis have been proved to be truly revertible, although suppressor mutation is well established. Such truly revertible alleles are necessary to verify for B. subtilis the suppressor-specificity of broth effect and MFD claimed for UV in E. coli (Bridges et al. 1967). It is improbable that of the 150 odd markers newly isolated in B. subtilis that some few will not show true reversion; the difficulty lies in their identification.

Classification of these markers according to their true or suppressor reversion would be considerably facilitated by the isolation of suitable phage mutants for testing. Bacterial revertants could then be tested for the presence of nonsense and certain missense suppressors by their ability to support the growth of phages bearing suppressible mutations. It is to be hoped that the increased research on B. subtilis phages in many laboratories means that such mutants will soon become available.

3. the autolysis shown by B. subtilis under certain cultural conditions is unfortunately not so easily circumvented. This means, effectively, that stationary phase cultures of the transformable strains cannot be used for mutation experiments. This limitation must be accepted as the price to be paid for transformation, a property which appears to be positively correlated with autolysis.

The advantages of B. subtilis on the other hand are two-fold and very considerable:

1. damage induced by alkylating agents in both E. coli and B. subtilis appears to be subject to cellular repair mechanisms. This much may be deduced from the isolation of alkylating agent-sensitive mutants of both species (Howard-Flanders and Boyce 1966, Strauss et al. 1966, Böhme and Geissler 1968). Only in B. subtilis, however, has the modification of mutation frequencies induced with alkylating agents been demonstrated. Broth effects have been described for EMS, DEB, MMS, and NTG in the present work, also MFD for EMS and NTG. With no alkylating agent has a broth

effect ever been found in E. coli. Strauss and Okubo (1960) show a stimulation of mutation yield in this organism by broth for DES and epichlorohydrin; this effect cannot be accepted as a genuine broth effect, however, as the comparison was drawn only with unsupplemented minimal medium without traces of the previous growth requirements. In addition, mutational damage induced by NTG in E. coli is repeatedly described as irreparable (Cerda-Olmedo and Hanawalt 1967, 1968, Hanawalt et al. 1968), also for B. subtilis (Yoshida and Yuki 1968), although it has been previously suggested that these last authors used an unsuitable plating medium. In the present work, however, MFD has been definitely described for B. subtilis after NTG treatment. Despite the achievement with E. coli in radiation mutagenesis, it seems possible that B. subtilis is the more suitable organism for the study of alkylating agent mutagenesis.

2. the main advantage of B. subtilis lies in its potential for genetic transformation, and it is around this special attribute that future work is planned.

It is all too frequently assumed that observed mutation frequencies represent the response of studied alleles to spontaneous or induced mutation. With the conspicuous exception of repair mechanisms, influences of genetic background or of conditions of treatment or assay are often disregarded. The exceptions presented in the INTRODUCTION to this thesis are sufficient to warrant further study of the mechanisms behind their exception.

It must be recognised that in observing recovery of

certain mutation frequencies, we are in fact studying the interaction of two types of process. The first of these is the primary reaction at the level of the DNA, whether brought about by a mutagen, or due to an inherent physico-chemical instability of a base. This reaction is a pre-requisite for mutation, although whether or not mutation actually results from it depends on the second process. This additional component comprises the secondary ancillary effects active on mutation induction, such as genetic back-ground, cellular physiology, environmental conditions, etc. (see Table 1 for examples). Unless the cumulative effects of these secondary processes are favourable, therefore, primary mutagenic reaction at the DNA level might either not be detectable at all, or at frequencies differing greatly from those of its induction. If we accept that the numbers and types of mutations recovered are determined by qualitative and quantitative cellular conditions, we must seek to prove it by separation of the various components.

It is at this level that the advantages presented by genetic transformation may be exploited. Functionally, the DNA may be treated with mutagen in vitro. and biologically assayed for mutation in recipient cells which have not been exposed to mutagen. By this means, the contribution of coincidentally treated cytoplasm may be eliminated, and nutritional effects on mutation expression studied without this veiled influence. The relevant control experiments will be the transformation of suitable wild-type markers in the same test-tube as the mutant assay. Further treatment

of recipient cells with the same or different mutagens as the DNA may, hopefully, throw light on the dose dependence of broth effects, and the mechanisms of mutational synergism. The genetic backgrounds of recipients may of course be varied at will, as may the phenotype. Added variation of the system will be permitted by DNA treatment in vivo, with extraction and assay of the DNA after various periods of post-treatment. Two factors have mediated against such an approach from the outset of this work - the complete absence of suitable mutational systems, and the relative inefficiency of the transformation process. The present work describes the elaboration of suitable systems for investigation.

Contemporarily with this work, refinements have been made by other workers with the biological assay system. These improvements include not only greater levels of competence for transformation of recipient cultures, but also the ability to reduce the heterogeneity of DNA preparations for which assay is desired. (e.g. Bott and Wilson 1968, Ayad, Barker, and Weigold 1968). To express this figuratively, if only a small fraction of cells in a recipient cell culture are able to take up DNA, then it is obviously better that they be offered a DNA preparation in which our studied marker is borne on one of ten types of DNA molecule than on one of two hundred. This reduction in heterogeneity of DNA preparations has been achieved in two ways - either by the fractionation of bacterial DNA by centrifugal or chromatographic techniques, or by "transfection".

"Transfection" is the name coined to describe the transformation of recipient cells with phage DNA, which, instead of recombination into the genome of the cell, now starts a normal phage infection. Some phages of B. subtilis have the singular advantage that their DNA may be isolated as a single "piece". Thus not only do we abolish any secondary effects of treatment, etc., on the recombination of bacterial markers into the genome of recipient cells, we also render unnecessary any recombination of phage DNA molecules for the formation of complete viral genomes.

It is only by analysis at this level that the nature of ancillary effects on mutation induction will be separated from supposition, and definitely elaborated.

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PUBLICATIONS

CORRAN J. 1967. "Mutation Frequency Decline" in Bacillus subtilis. I. Ethyl methanesulphonate as mutagen. II. Ultraviolet light as mutagen. Microbial Genetic Bulletin. 26. 5-6.

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"Mutation frequency decline" in Bacillus subtilis. I. Ethyl methanesulphonate as mutagen.

When a suspension of Bacillus subtilis cells was treated with ethyl methanesulphonate - 40 minutes, 37'C, 0·075 M. - suppressor mutants were obtained in which 2 of the previous auxotrophic requirements had been abolished. The strain of Bacillus subtilis used was SB.5. from the Nester collection, to which an ultraviolet-light-induced leucine requirement had been added, i.e. leu^- his_1^-, ura^- try_2^-. The mutants scored were jointly independent of leucine and histidine, and were obtained in highest yield when the selection medium was prewarmed minimal agar + glucose + uracil and tryptophan (30 µg/ml each) + 1% v/v "Oxoid" Nutrient Broth (M + B). Mutants could also be recovered in lower yield on minimal agar + glucose + uracil and tryptophan (30 µg/ml each) + leucine and histidine (1 µg/ml each) (M + S). If plating on these selective media was delayed by suspension and incubation of the cells in liquid minimal medium + glucose at 37'C, the number of mutants recoverable on M + B medium decreased as the incubation period was lengthened, only 50% (circa) of the mutants obtainable on immediate plating being recovered after 45 minutes preplating incubation, and 25% (circa) after 75-80 minutes incubation. During this pre-plating incubation in minimal medium + glucose, total viable cells showed no decrease, and the number of mutants recoverable

on M + S medium remained constant, or showed only a very slight decrease in no way comparable with that shown on broth-supplemented plates.

If the 4 growth requirements of the strain were supplied during this preplating incubation in minimal medium + glucose, or alternatively if addition of 1% v/v "Oxoid" Nutrient Broth was made, the decrease with time of incubation of mutants recoverable on M + B medium was very considerably reduced, also the number of mutants recoverable on M + S medium rose with increasing preplating incubation time until it reached the number obtainable on M + B medium.

It is believed that this represents "Mutation Frequency Decline" (M.F.D.) of mutants recoverable on M + B medium on incubation in liquid minimal medium + glucose. This M.F.D. may be partially prevented by the addition of the auxotrophic requirements (or broth) which allow fixation/expression of the mutants so that the yield obtained in M + S medium approached that on M + B medium with increasing length of incubation.

II. Ultraviolet light as mutagen. Using the same strain ($leu^- his_1^- ura^- try_2^-$) of Bacillus subtilis, a drastic decrease was observed in the number of mutants scored after a mutagenic dose of ultraviolet allowing high survival. The system as studied so far is not directly comparable with the ethyl methanesulphonate mutation system described in Part I, as a) UVirradiated cells on membrane filters were held on Nutrient Broth Agar for 2 hours and then selected as

individual histidine or leucine 'revertants'; b) if the cells were held on minimal agar + glucose before this 2 hour period on Nutrient Broth Agar, the number of mutants scored decreased with increased time of such holding, whether or not the 4 required supplements were present. The systems are being further studied. ------ M.R.C. Mutagenesis Research Unit, Institute of Animal Genetics, West Mains Road, Edinburgh, Scotland.

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pp. 8-9.

Corran J.

Mutation Frequency Decline following nitrosoguanidine treatment of Bacillus subtilis.

The induction by N-methyl-N-nitroso-N'-nitroguanidine (NG) of supersuppressor mutants of Bacillus subtilis showing the simultaneous relief of histidine and leucine requirements has been studied. NG treatments were at 25 µg/ml, 37°C, for varying times with removal by dilution and centrifugal washing of cells. Mutation yields at all doses were dependent on the degree of supplementation of the selective medium. Higher mutation frequencies per survivor were recovered on agar with 1·5% v/v nutrient broth (br-agar) than on agar with 1·5 µg/ml each of histidine and leucine, the previously required growth supplements (hl-agar). The smallest yield of mutants occurred on unsupplemented minimal agar. Mutation Frequency Decline (MFD) took place on solid media only in the absence of broth, but in liquid media regardless of the presence of broth.

Viable counts were also scored on three media, br-, hl-, and nutrient agars. Immediately after NG treatment, viable counts were much less (c.50%) on hl- and nutrient agars than on br-agar. During a posttreatment preplating incubation in liquid medium, these counts rose to the levels found on br-agar. This suggests that hl- and nutrient agars

are suboptimal for scoring cells immediately after NG treatment. With the exception of these variations in viable count, the results are similar to those reported using ethyl methanesulphonate (EMS) as mutagen (Corran, Molec. Gen. Genetics 1968 103 (2) 42).

This finding may have a bearing on a recent report of NG mutagenesis with a phe^- mutant of Bacillus subtilis (Yoshida and Yuki Japanese J. Genetics 1968. 43. 173) where a recovery of survival of NG-treated cells scored on nutrient agar was described during a posttreatment incubation in liquid medium. Although inhibited by acriflavine, this 'recovery' may represent the adaptation of NG-treated cells to suboptimal plating media. These workers also reported no change in mutation frequency to phe^+ during this liquid incubation which contrasts with the MFD reported here fur supersuppressor mutants. As they used for mutant assay an unsupplemented minimal agar, it is possible that their observed mutation frequencies are 'base-line' figures, the lowest possible mutation frequency being recovered on this medium regardless of any intermediate incubation in liquid. Alternatively, the different behaviour of supersuppressor and phe^+ mutants may represent differences in response to medial supplementation of suppressor and true revertants, as was found with UV-induced try^+ mutants of a strain of Escherichia coli (WP2). The kind gift of a culture by Dr's. Yoshida and Yuki will make it possible to compare supersuppressor and phe^+ mutants in the EMS-mutagenesis system. -------- M.R.C. Mutagenesis Unit, Department of Genetics, University of Edinburgh, Scotland.

Molec. Gen. Genetics 103, 42—57 (1968)

The Induction of Supersuppressor Mutants of *Bacillus subtilis* by Ethyl Methanesulphonate and the Posttreatment Modification of Mutation Yield

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Summary. Supersuppressor mutants have been induced in a strain of *Bacillus subtilis* with the chemical mutagen ethyl methanesulphonate. The yield of mutants recovered is dependent on the degree of supplementation of the selective plating medium with minute quantities of either nutrient broth or the previously required growth supplements. The optimal quantities of these medial additives have been established and the superiority of nutrient broth described. This "broth effect" has been shown to be due to components of the nutrient broth other than the previously required growth substances.

This stimulatory effect of nutrient broth on mutation yield is completed after approximately 2 hours incubation on solid medium. Conversely, absence of broth during the first two hours of incubation on solid medium leads to a time-dependent, irreversible decline in mutation frequency even when incubation is continued upon selective agar with added broth. In liquid media, mutation frequency decline takes place in a manner similar to that observed on solid media, but the stimulatory effects of broth upon mutation fixation and mutant recovery are no longer evident. A decline in mutation frequency occurs in liquid of any composition, but addition of leucine and uracil reduces its degree. It is suggested that the additional phenomena detected in liquid media are due to a liquid holding recovery, although the removal of residual, as yet unreacted, mutagen or mutagenic intermediates into liquid cannot be ruled out.

Introduction

Studies on induced mutation in bacteria have demonstrated that the yield of mutants obtained is frequently dependent upon the composition of the selective plating medium, and upon the nature of treatments applied to the cells between mutagen treatment and plating. Two closely related phenomena have been described for ultraviolet-induced reversion to prototrophy in *Escherichia coli* — a "broth effect", detected as an enhancement of mutation yield on enrichment of the plating medium with a small quantity of nutrient broth or some other source of an amino acid pool, and "mutation frequency decline", where potential mutants are irreversibly lost when the treated cells are discouraged from protein synthesis.

Until recently it has not been possible to examine the nature of the mutants obtained as the strains most commonly used, *Escherichia coli B*, *B/r*, and their derivatives, have not been suitable for the techniques of genetic analysis necesary to detect whether suppressor mutations, nonsuppressor mutations, or a mixture of these two broad classes have arisen. It has been reported by WITKIN (1963) that suppressor mutations may be induced with UV-irradiation in strains of *Escherichia coli* which show the simultaneous relief of one or more auxotrophic

requirements of independent origin; these suppressor mutations were also reported to be sensitive to post-mutagenic treatments of short duration resulting in an irreversible decline in induced mutation frequency.

The recently devised technique for classifying some bacterial revertants by the use of "nonsense" mutants of bacteriophage T 4 has been utilised to demonstrate that revertants to prototrophy of *Escherichia coli B/r try*$^-$ were of both suppressor and nonsuppressor classes, this last class presumably including true reversions (BRIDGES, DENNIS and MUNSON, 1967a; OSBORN and PERSON, 1967). Furthermore, it has been shown that those revertants of this strain of *Escherichia coli* that responded to medial supplementation and posttreatments in which protein synthesis was discouraged appeared to be overwhelmingly of the suppressor class (BRIDGES, DENNIS and MUNSON, 1967b).

Most of the information available concerning the response of microbial mutagenesis to posttreatment modification has been gathered with a strain of *Salmonella typhimurium* and strains of *Escherichia coli*, especially derivatives of *B/r*, subjected to mutagenic irradiation, most commonly in the ultraviolet. In this communication it is reported that supersuppressor mutants are inducible in *Bacillus subtilis* with an alkylating agent as mutagen, that the yield of mutants is dependent upon the degree of supplementation of the plating medium, and that a process analogous to the "mutation frequency decline" already described occurs in the induced mutant fraction of the population of treated cells. In addition, discrepancies are described in the behaviour of treated cells in liquid medium and on solid medium. The isolation of supersuppressor mutants of *Bacillus subtilis* from late logarithmic phase cultures has been previously described in a short communication (CORRAN, 1967).

Materials and Methods

1. Bacterial Strain

The strain used throughout these experiments was a quadruple auxotroph of *Bacillus subtilis*. The strain was received from Dr. W. DONACHIE as *Bacillus subtilis* SB5 his_1^- try_2^- ura_1^-, since renamed *Bacillus subtilis* BD2 *hisA 1*$^-$ *ura-1*$^-$ *try-2*$^-$ following DUBNAU, GOLDTHWAITE, SMITH and MARMUR (1967). The fourth requirement, for leucine, was selected in this laboratory following ultraviolet mutagenesis, enrichment growth, penicillin treatment, and standard auxanographic techniques.

2. Media

The basal minimal salts solution was that described by SPIZIZEN (1958) which served also as diluent and wash fluid for the cells. When used as a liquid, $MnCl_2$ was added to 10^{-6} M, and as solid or liquid growth media, glucose to 1 and 0.5% w/v respectively. Solid medium was prepared by the addition of Davis New Zealand Agar to 1.5% w/v. L-histidine, L-leucine, L-tryptophan, and uracil (from British Drug Houses Ltd.), were kept as sterile aqueous solutions at 2 mg/ml and added as required. Casein hydrolysate (Nutritional Biochemicals Corp., acid, salt-free) was kept in sterile aqueous solution at 2% w/v, and used at a final concentration of 0.02% w/v. Agar and water, glucose, 5× concentrated salts solution and other supplements were separately sterilised and mixed only upon cooling.

For a complete medium, nutrient broth (Oxoid Ltd., London) was used, as liquid, or solidified by the addition of 1.5% w/v Davis New Zealand Agar.

Abbreviations used in the text: EMS = ethyl methanesulphonate, MMS = methyl methanesulphonate, UV = ultraviolet irradiation, MFD = mutation frequency decline.

3. Preparation of Cultures

A small inoculum was transferred from a stock nutrient agar plate to 50 ml of liquid minimal salts and glucose medium supplemented with 30 μg/ml of each of the four growth requirements and casein hydrolysate at 0.02% w/v. The Erlenmeyer flask containing the inoculated medium was shaken for 15—16 hours in a 37° C water bath and the cells then diluted at least $^1/_{20}$ into fresh liquid minimal salts and glucose medium supplemented with 30 μg/ml of each of the four growth requirements without added casein hydrolysate. After 3—4 hours shaking in the water bath at 37° C, the rapidly growing culture, containing $1—3 \times 10^8$ cells per ml, was harvested by centrifugation at 15,000 g for 5 minutes and resuspended in approximately $^1/_{10}$ volume liquid minimal salts medium for treatment.

4. Mutagen Treatment

Ethyl methanesulphonate (Eastman Organic Chemicals) was dissolved in liquid minimal salts medium before use, and a quantity of this solution added to the cell suspension to give the desired concentration of 0.075 M. The mixture was then shaken gently at 37° C.

To terminate the mutagenic treatment, sodium thiosulphate solution was added to 2% w/v, the suspension centrifuged at 20,000 g for 5 minutes, and the pellet of cells taken up and centrifugally washed twice in liquid minimal salts medium containing sodium thiosulphate at 1% w/v. The final pellet of cells was resuspended in liquid minimal salts medium to the desired concentration and either plated at once or subjected to various preplating treatments. In all experiments here described, other than those in Results I, a single uniform dose of 40 minutes, 37° C, 0.075 M EMS was given.

5. Counts of Mutants and Survivors

Mutants to *hisA 1⁻ leu⁻ try-2⁻ ura-1⁻* $su^+_{hisA1,leu}$ were scored after 4 days incubation at 37° C on three different media; a) selective agar = minimal salts and glucose agar with 30 μg/ml each of uracil and tryptophan, b) Selective agar as in a) further supplemented with trace quantities of the previously required growth factors, histidine and leucine, usually 1.5 μg/ml of each, c) selective agar as in a) further supplemented with small quantities of nutrient broth, usually 1.5% v/v. The experiments in which these optimal quantities of further supplements were established are described in Results II.

The total viable count was scored after 2 days incubation at 37° C also on three different media; a) minimal salts + glucose agar + 30 μg/ml of each of the 4 growth requirements. b) and c) were the media described above as mutant selection media b) and c). Whenever possible, mutants and survivors were scored on agars of equal supplementation. The viable count as estimated did not increase between 2 and 4 days incubation. For each plating, 0.1 ml aliquots were pipetted on to three plates of each solid medium for each relevant dilution used for a particular sample and gently spread over the surface of the agar with a bent glass rod. The agar used for plating was prewarmed to 37° C, and following the rapid absorption of the inoculum, the plates were replaced at 37° C. Comparable results were obtained when the aliquots of inoculum were plated in a top layer of agar.

A control suspension, untreated with EMS, was included in each experiment. Mutants of the selected phenotype arose spontaneously with infrequency, and their number was subtracted from the number of induced mutants per plate.

In some experiments, the aliquots of suspension were spread or impinged on the surface of Oxoid 6 cm diameter filter membranes on the relevant agar. This procedure allowed the removal of plated cells from one medium to another at selected times by simply moving the filter membrane from one agar plate to another.

It should be noted that of several hundred mutant colonies tested from several experiments, originally isolated on minimal salts + glucose medium supplemented with 30 μg/ml each of uracil and tryptophan and trace quantities of histidine and leucine or nutrient broth, not one proved incapable of growth on minimal salts + glucose agar supplemented only with 30 μg/ml each of uracil and tryptophan. This demonstrates that the extra mutants obtained on these further supplemented media are not incapable of growth on the simpler media and that the difference is one of mutation expression.

Results

1. Mutation Induction

During mutation studies with the *hisA 1* marker in a number of genetic backgrounds, it was discovered that when the *hisA 1* marker and a specific leucine marker were present in the same strain, supersuppressor mutants independent of both requirements arose spontaneously at low frequency. This frequency was very much enhanced if the strain was treated with EMS or MMS and plated on a selective minimal medium, particularly when this medium was enriched with trace quantities of nutrient broth or of the previously required growth factors, histidine and leucine. Supersuppressor mutants have also been obtained after UV-mutagenesis, although in this case a different recovery technique must be used (unpublished results).

Table 1 shows the results obtained from one such experiment with EMS. Survivals, which were high, are not given as they depend not only upon the

Table 1. *Induction of mutants by EMS and their recovery on different media*

Minimal salts + glucose agar supplemented with:	Time of exposure of cells to EMS in minutes (0.075 M, 37°C)						
	10	20	30	40	50	60	70
30 μg/ml each uracil and tryptophan	1.05 10	24 220	80 480	136 1,060	338 1,910	520 3,110	— —
30 μg/ml each uracil and tryptophan + 1.5 μg/ml each of histidine and leucine	12 120	118 1,100	491 3,240	811 5,840	1,246 7,100	1,947 11,100	2,359 16,750
30 μg/ml each uracil and tryptophan + 1.5% v/v nutrient broth	20 220	398 4,450	1,795 14,900	2,577 25,000	4,760 37,600	6,979 49,550	7,410 53,350
"Broth Effect"	1.66	3.37	3.66	3.18	3.82	3.58	3.14

The upper figure in each square is the number of mutants induced per 10^8 survivors. Mutants and survivors were scored on the same type of agar, except where mutants were scored on agar + uracil and tryptophan only, when the viable count was scored on agar + all 4 growth requirements at 30 μg/ml each. The lower figure in each square is the number of mutants scored per ml. of suspension plated corrected for any spontaneously arising mutants. The "broth effect" is the mutation frequency on broth-supplemented agar divided by the mutation frequency on agar supplemented with traces of the previous growth requirements at each dose.

lethality of the treatment, but also upon the recovery of cells of each sample during separate centrifugal washing procedures. In the experiment shown in Table 1, survival on minimal salts + glucose agar supplemented with uracil and tryptophan (at 30 μg/ml each) and 1.5% v/v nutrient broth after 70 minutes EMS treatment was 57.6%. However the essential comparison lies not between doses but between different media at each dose. Survival does not differ substantially when measured on the three different media used for this purpose although counts were sometimes fractionally higher on broth-supplemented agar. The results demonstrate the increase of induced mutation frequency with longer exposure times of the cells to the mutagen, and it is also evident that the addition

of 1.5 μg/ml each of histidine and leucine to the selective agar permitted further mutation expression and an enhanced mutation frequency. Addition of 1.5% v/v nutrient broth allowed an even greater enhancement of induced mutation frequency than 1.5 μg/ml each of histidine and leucine. The magnitude of this broth-enhancement lay between 3 and 4 fold although it was dose-dependent and did not reach the maximal range until the 20-minute exposure to EMS.

2. Dependence of Induced Mutation Frequency on the Degree of Supplementation of the Selective Medium

The greater yields of mutants obtained on selective agars with traces of further enrichment indicate that conditions favourable to residual synthesis,

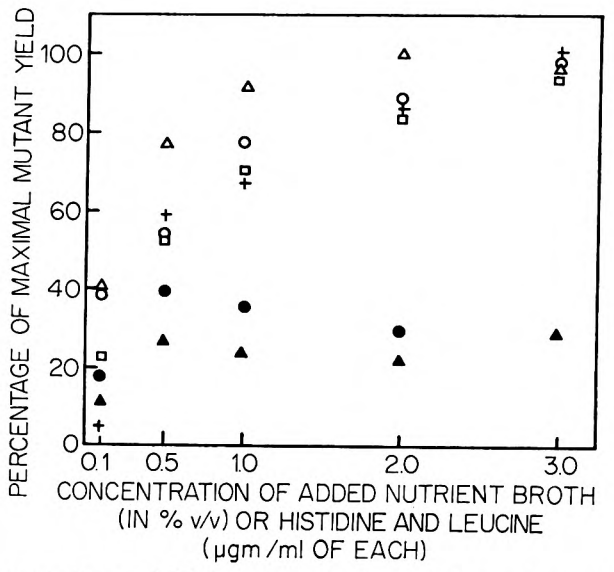


Fig. 1. The response of mutation yield from a single EMS treatment to increasing supplementation of the selective agar with nutrient broth (+ ○ □ △ separate experiments) or histidine and leucine (● ▲ separate experiments)

even division, on the agar may be responsible for the enhancement, and that the increased yields obtained on broth-supplemented agar compared with agar supplements with traces of the previously required growth factors might be caused simply by a histidine and leucine content in 1.5% v/v nutrient broth greater than 1.5 μg/ml of each amino acid, the "trace quantities" added to the selective agar. Alternatively the extra enhancement noted with broth supplementation might be due to components of the broth other than histidine and leucine.

A series of replicate experiments was then performed in which suspensions of cells were given an EMS-treatment and plated on selective agars enriched with varying concentrations of nutrient broth or of histidine and leucine. The results of these experiments are given in Fig. 1. While there was variation between experiments, it is clear that the maximal yield of mutants in each experiment on selective agar enriched with traces of the previously required growth factors was obtained when the concentration of histidine and leucine was 0.5 μg/ml of each amino acid, and that an increase in this concentration of 4—6 times did not increase the yield of mutants. This indicates that in the mutation induction experiments, of which that given in Table 1 is an example, the reduced yield of

mutants obtained on selective agar enriched with traces of the previously required growth factors compared with broth-supplemented selective agar is not due to the 1.5 μg/ml each of histidine and leucine present being limiting, since an increase in this concentration does not increase the mutant yield. Constituents of nutrient broth other than histidine and leucine are therefore responsible for this extra enhancement. Fig. 1 also illustrates that enhancement of mutation yield with broth or histidine and leucine addition to selective agar begins with a rapid increase in mutation yield, larger with broth; the broth enhancement continues up to high levels whilst the histidine and leucine enhancement shows no further increase after 0.5 μg/ml of each amino acid. This "broth effect" is very similar to that noted by WITKIN (1956), MUNSON and BRIDGES (1966) and CLARKE (1967a, b) for UV-induced mutation in some strains of *Escherichia coli* and a strain of *Salmonella typhimurium*. It has also been shown by DOUDNEY and HAAS (1958) that an enhancement in UV-induced mutation frequency of *Escherichia coli* can be obtained with a preplating posttreatment in amino acid-rich medium.

The optimal concentration of nutrient broth supplementation in the selective agar is 2—3% v/v, and results not given here indicate that there is only a fractional increase up to 5% v/v supplementation. Survival does not differ appreciably on selective agars with 0.1—5.0% v/v nutrient broth or 0.5—5.0 μg/ml each of histidine and leucine; microcolonies on selective agar with 0.1% v/v nutrient broth must be scored with the aid of a lower power dissecting microscope. As might be expected, the residual growth of the nonmutated cells in the inoculum increased with the concentration of supplements added, forming a thick lawn of growth at 3% v/v nutrient broth and over, and at 4—5 μg/ml each of histidine and leucine. To facilitate the scoring of mutant colonies, concentrations of supplements added were limited in subsequent experiments to 1.5% v/v nutrient broth and 1.5 μg/ml each of histidine and leucine, allowing respectively c. 80—90% and c. 100% recovery of mutants obtainable on the two types of media.

3. MFD on Solid Media

In the classical work of WITKIN (1956) on UV-mutagenesis of strains of *Escherichia coli* and *Salmonella typhimurium*, she demonstrated the value of "switch-experiments" in which the treated cells were incubated for a time on one medium with final selection on another, the difference between the media being of degree of supplementation. It was of interest to determine the response of these EMS-induced supersuppressor mutants of *Bacillus subtilis* to such medial modification.

In several experiments, aliquots of uniformly treated cells were spread on Oxoid filter membranes on the surface of plates containing either broth-supplemented agar or agar supplemented with traces of the previous growth requirements. After 2 hours of incubation at 37° C, the membranes were either left undisturbed, or changed to a fresh plate containing agar of the same or different supplementation, with incubation continued up to 4 days. Similar experiments were performed using as first medium agar with an excess of the four growth requirements (30 μg/ml each) or selective agar with 1.5 μg/ml each of histidine

and leucine, and as second selective medium agar supplemented with traces of broth or of histidine and leucine. Results are given in Table 2.

Experiments I and II demonstrate clearly the higher mutant yield obtained on broth-supplemented agar, also that two hours on this medium was sufficient to render the mutants immune from MFD on broth-free selective agar. It is apparent from experiments I—IV that a process of MFD took place on broth-free agars regardless of the concentration of specific growth requirements present. Complete medium (nutrient agar) was almost as effective as broth-supplemented selective agar when used as medium I in such experiments.

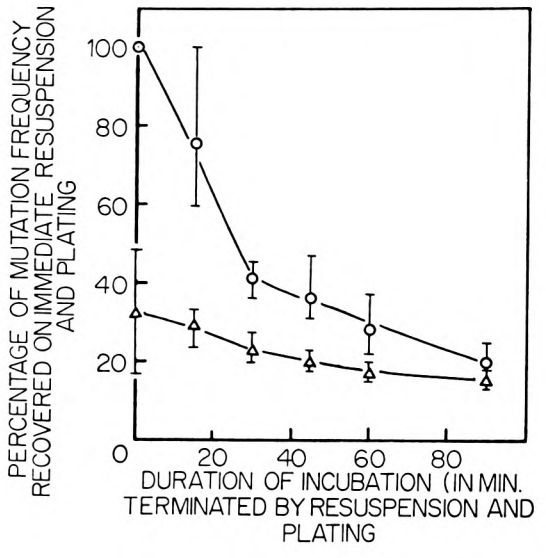


Fig. 2. MFD during incubation of populations of EMS-treated cells on filter membranes on minimal salts + glucose agar at 37° C with resuspension and plating at intervals for assay of mutant and total viable counts. ○ Mutant yield on selective agar + 1.5% v/v broth. △ Mutant yield on selective agar + 1.5 μg/ml each of histidine and leucine. (Each point is derived from the mean of at least 3 experiments. Horizontal bars are drawn between the extremes of experimental means)

Table 2. *Mutation fixation and mutation frequency decline on solid media*

Agar medium I (duration of incubation 2 hours)	Agar medium II (incubation continued until scoring)	Mutants per membrane (mean of three membranes)			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
Selective agar + broth	Selective agar + broth	275	82	—	—
Selective agar + broth	Selective agar + traces of histidine and leucine	251	98	—	—
Selective agar + traces of histidine and leucine	Selective agar + broth	53	36	57	85
Selective agar + traces of histidine and leucine	Selective agar + traces of histidine and leucine	55	23	50	66
Agar + the four growth requirements in excess	Selective agar + broth	—	—	60	66
Agar + the four growth requirements in excess	Selective agar + traces of histidine and leucine	—	—	32	61
Selective agar + broth throughout		289	90	181	231
Selective agar + traces of histidine and leucine throughout		30	26	46	58

Cells on filter membranes were changed from agar medium I to agar medium II at 120 min, 37° C. Selective agar = minimal salts + glucose agar with uracil and tryptophan each at 30 μg/ml. Broth = 1.5% v/v nutrient broth. Traces of histidine and leucine = 1.5 μg/ml each of histidine and leucine. The 4 growth requirements in excess = histidine, leucine, tryptophan and uracil each at 30 μg/ml.

In a further series of experiments, treated cells were spread on to filter membranes and held on the surface of minimal salts + glucose agar plates, with resuspension and plating at intervals. A rapid decline in mutation frequency occurred of mutants recoverable on broth-supplemented selective agar, after 90 minutes on minimal salts + glucose agar only 20% of the mutants being recoverable. A small but definite decline also occurred of mutants scoreable on selective agar supplemented with traces of the previous growth requirements, as shown in Fig. 2. The observance of this MFD on filter membranes on the surface of minimal salts + glucose agar plates from which excess moisture had been removed renders unlikely any explanation of the MFD based totally on a "washing-out" from the cells of residual mutagen or mutagenic intermediates.

4. MFD in Liquid Media

The discovery of this medial-dependent MFD on solid media was followed by experiments in which the treated cells were suspended in various liquid media before final selective plating. In Fig. 3 are shown results from several experiments in which the mutagen-treated cells were incubated for varying periods in liquid minimal salts + glucose medium (black symbols, broth-supplemented agar continuous line, agar supplemented with traces of previous growth requirements broken line). A dramatic MFD is evident, in 100 min only 11% being recoverable of the mutants arising on broth-supplemented agar at 0 min, and about 30% of those mutants recoverable on agar supplemented with traces of the previously required growth factors. Overall survival of the culture showed no decline at all.

In contrast to the results of experiments with solid intermediate media, it was unexpectedly found that addition to liquid minimal salts + glucose medium of uracil and tryptophan (30 μg/ml each) and 1.5% v/v nutrient broth did not stop the MFD regardless of the final selective plating medium. The MFD curves in the liquid equivalents of the selective agars (with or without broth or traces of the previous growth requirements) follows very closely, although always slightly above, that of MFD in liquid minimal salts + glucose medium. Addition of nutrient broth to a concentration as great as 50% v/v proved unable to stop the MFD although a small reduction was observed.

However the composition of the suspending liquid is not irrelevant to the inhibition of MFD. A posttreatment performed in liquid minimal salts + glucose medium supplemented with 30 μg/ml of each of the growth requirements of the strain gave a modified MFD curve as shown in Fig. 3 (open symbols, broth-supplemented agar continuous line, agar supplemented with traces of previous growth requirements broken line). In the presence of the four growth requirements, both the rate and extent of the decline of mutants scoreable on broth-supplemented selective agar were considerably reduced whilst that of mutants scoreable on selective agar supplemented with traces of the previous growth requirements was completely abolished. A similarly reduced MFD was obtained when the posttreatment was performed in liquid minimal salts + glucose medium supplemented with 30 μg/ml each of uracil and tryptophan and 0.02% w/v casein hydrolysate, or with 30 μg/ml each of the four growth requirements and 1.5% v/v

nutrient broth. Experiments have shown that only two components of these various supplements are necessary for the reduction in MFD, as the addition of leucine and uracil alone at 30 μg/ml was sufficient to produce the effect, even when the cells had been starved of histidine and tryptophan before mutagen treatment. As this starvation period should reduce the intracellular 'reserves' of histidine and tryptophan, it is unlikely that the MFD-sparing effect of leucine and uracil is due to the combination of an exogenous supply of these two requirements coupled with an endogenous supply of histidine and tryptophan with much resultant synthesis.

In contrast to the addition of leucine and uracil, nutrient broth at concentrations as great as 50% v/v had only a marginal effect on MFD in liquid media

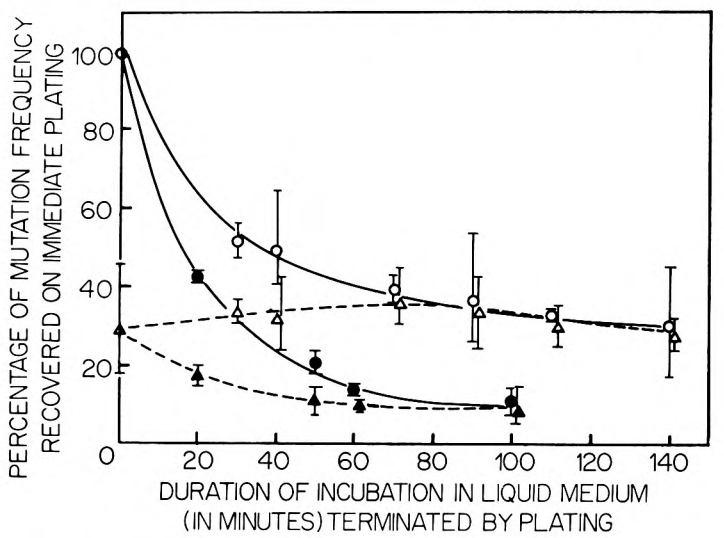


Fig. 3. MFD during incubation of EMS-treated cells in liquid minimal salts + glucose medium without enrichment (● ▲) or with added leucine, uracil, histidine and tryptophan at 30 μg/ml each (○ △) with plating at intervals for assay of mutant and total viable counts. ● ○ Mutation yields on selective agar + 1.5% v/v broth, ▲ △ Mutation yields on selective agar + 1.5 μg/ml each of histidine and leucine. (Each point is derived from the mean of at least 3 experiments. Horizontal bars are drawn between the extremes of experimental means)

of mutants recoverable on selective media with or without broth supplement. Since 1.5% v/v nutrient broth does not allow MFD on solid media, the difference must lie in the response of treated cells to the two different states, liquid and solid, or to the materials involved in the hardening of the solid state. It is possible, for example, that in these two states cell-medium diffusion differs, that different osmotic values obtain, or that an agar component in company with broth may produce a high mutation yield from treated cells. Experiments were then performed to test the influence of three factors on MFD, the omission of various components from the liquid used for intermediate incubation, the osmotic stabilisation of this intermediate liquid, and the attempted extraction of active impurities from the agar itself.

Media from which components were separately or jointly omitted, *i.e.* glucose, ammonium sulphate, sodium citrate, or simple suspending liquids such as 0.85% w/v saline or phosphate-buffered saline, gave a slight sparing of MFD but none of these liquids was as effective in this regard as the leucine and uracil-enriched

liquid minimal salts + glucose medium already described. Some slight reduction in viability occured in most omission media. The greatest MFD-sparing was shown by liquid minimal salts medium without both citrate and glucose, suggesting that the processes giving MFD in liquid media, whilst not requiring an energy source, act at maximal efficiency in the presence of one. Attempted osmotic stabilisation by addition of sucrose to 20% w/v or sodium chloride to 0.5 M showed only fractional sparing of MFD. Several attempts were made to extract a postulated agar component whose absence might explain the MFD observed in broth-supplemented liquid medium. Concentrated agar gels were exposed for many days to liquid minimal salts + glucose medium containing uracil and tryptophan at 30 μg/ml each and nutrient broth at 1.5% v/v, then the liquid medium was filtered off and used as suspension medium for mutagen-treated cells. Although it was obvious from the pale straw colour of the liquid medium that substances were being eluted from the agar gels, MFD proceeded normally in the eluent liquid medium with its broth supplement. In a complementary experiment when a purer agar (Oxoid Immunodiffusion Agar) was used instead of the usual Davis New Zealand Agar, induced mutation frequency showed the same medial dependence and MFD proceeded normally.

In addition, caffeine at 0.5 mg/ml or a mixture of non-specific amino acids (glutamic acid, aspartic acid and proline at 30 μg/ml each) proved powerless to stop MFD in broth-supplemented liquid medium.

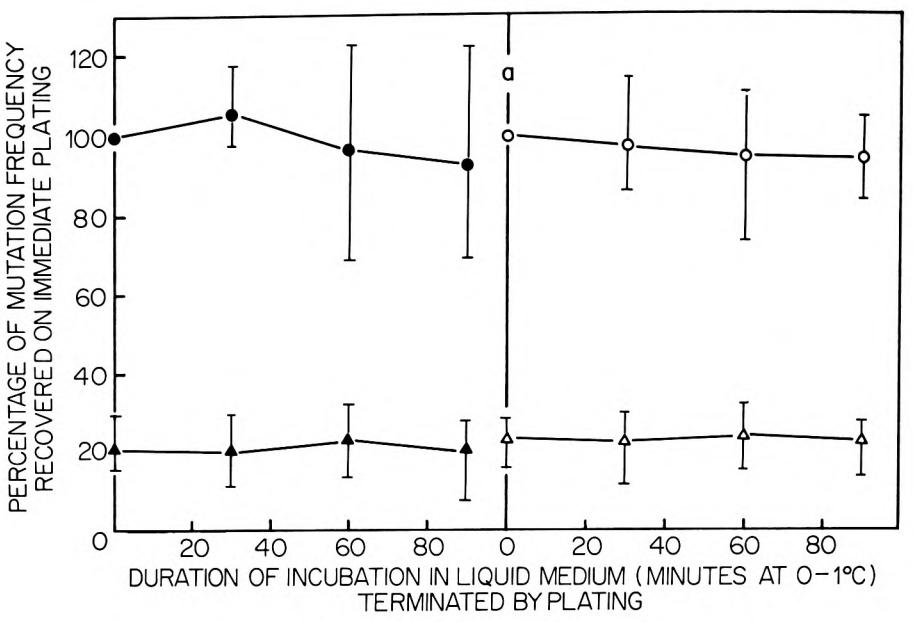
5. Effect of Temperature on MFD in Liquid Media

Experiments preformed at 20 and 37° C showed that MFD was only slightly reduced at the lower temperature. If the treated cells were shaken at 0—1° C in a melting ice bath, MFD was inhibited in all media used, as illustrated in Fig. 4a. If the cells were removed at intervals from the low temperature and incubated at 37° C, MFD took place in the usual manner as shown in Fig. 4b.

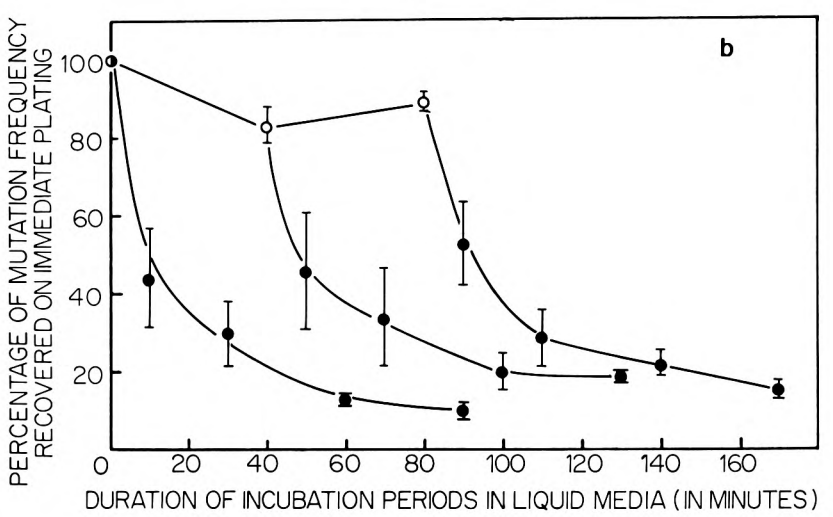
6. Effect of Storage as a Centrifuged Pellet

It has been demonstrated previously that after cessation of direct mutagenic treatment of conidia of *Neurospora crassa* with diepoxybutane or ethylene oxide, induced mutation frequency increased if the conidia were stored at 22—30° C in the form of a centrifuged pellet. Plating or vigorous shaking of the conidia in water at 22—30° C rapidly eliminated this "after-effect" which has been ascribed to residual mutagen inside the conidia. This residual mutagen was presumed to be immune from centrifugal washing procedures, but to be removed from the conidia by vigorous shaking in water and inactivated on plating, perhaps by the dilution involved (Kølmark and Kilbey, 1962; Kilbey and Kølmark, 1968).

Such residual mutagen or mutagenic intermediates, immune from thiosulphate — washing procedures, might be responsible for the phenomena described for *Bacillus subtilis*, and an investigation was made of the effect of storage of treated cells as centrifuged pellets in the bottom of centrifuge tubes at 0° C and at 37° C. Cells were treated with EMS in the normal manner, thiosulphate-washed, and either plated immediately or after 2 hours storage following resuspension of the pellets. Results from two such experiments are given in Table 3.



a



b

Fig. 4a and b. Effect of temperature on MFD in liquid media. a. Inhibition of MFD at 0—1° C in liquid minimal salts + glucose medium without enrichment (● ▲) or with added casein hydrolysate to 0.02% w/v and tryptophan to 30 μg/ml (○ △) with plating at intervals for assay of mutant and total viable counts. ● ○ Mutation yields on selective agar + 1.5% v/v broth. ▲ △ Mutation yields on selective agar + 1.5 μg/ml each of histidine and leucine. b. Commencement of MFD after inhibition at 0—1° C on raising incubation temperature to 37° C. Plating medium = selective agar + 1.5% v/v broth and the incubation liquid = liquid minimal salts + glucose medium, at 0—1° C (○) or at 37° C (●)

Whilst no MFD took place in the pellets at 37° C or at 0° C, no increase in mutation frequency was observed during storage at either temperature. Lethality increased in the pellets held at 37° C in a manner similar to that of untreated control cells.

Discussion

Solid Medium

It is shown in Fig. 1 that the stimulation of EMS-induced mutation yield in *Bacillus subtilis* effected by the addition of small quantities of nutrient broth to the selective medium, the "broth effect", is not due simply to the provision

of extra quantities of the required growth factors with consequently greater mutation expression. It is evident that maximal mutation expression in the presence of the previously required growth factors, histidine and leucine, occurred at concentrations of 0.5 μg/ml of each amino acid, and that an increase in these concentrations of 4—6 times produced no corresponding increase in mutation yield. Differential survival upon the different media does not provide a probable explanation either, as the survival of EMS-treated cell populations was similar upon the three types of plating media employed after the extra dilution necessary for viable counting. Survivals on the three media were also similar in the filter membrane experiments when viable counts were made after changes in mutation frequency had occurred. The "broth effect" must therefore be caused by components of the nutrient broth other than the histidine and leucine previously required by the mutants. This "broth effect" is similar to that noted by WITKIN (1956), MUNSON and BRIDGES (1966) and CLARKE (1967a, b) for the UV-induction of mutation in a strain of *Salmonella typhimurium* and strains of *Escherichia coli*, and it demonstrates clearly the modification of induced mutation frequency by components of the plating medium other than any quantities of the previously required growth factors needed for mutation expression.

As may be seen in Table 2, the "broth effect" was completed within two hours as transfer of the cells at this time to media lacking broth resulted in a mutation frequency characteristic of broth-supplemented agar. Incubation of EMS-treated cells for two hours upon agar lacking broth with subsequent transfer to broth-supplemented agar for final incubation on the other hand yielded a mutation frequency characteristic of media lacking broth. During this period of two hours therefore, mutants were irreversibly lost, not to appear on addition of broth even when the medium used for intermediate incubation contained excess quantities of all the growth requirements by the strain. A similar dependence on time and broth inclusion in the medium was found by WITKIN (1956) and DOUDNEY and HAAS (1958) with UV-induced mutations of *Salmonella typhimurium* and *Escherichia coli*.

The kinetics of MFD shown in Fig. 2 demonstrate that the yield of mutants recoverable on broth-supplemented agar declined as the time of intermediate incubation of the cells on minimal salts + glucose agar without broth increased. A small decline of mutants recoverable on agar supplemented with traces of the previously required growth factors was also evident. This MFD was therefore inhibited on solid media by the presence of small quantities of nutrient broth. These results show the same three post-mutagenic events involved in mutation induction by UV in *Escherichia coli* (DOUDNEY and HAAS, 1958): cells held under conditions unfavourable to macromolecular synthesis show an immediate decline in mutation frequency (MFD), whereas conditions favourable to macromolecular synthesis induce a process of "mutation stabilisation" terminated by "mutation fixation". Imposition of unfavourable conditions during "mutation stabilisation" causes MFD, but once the cells have attained "mutation fixation" they are immune from such medial vagaries. As DOUDNEY and HAAS (1960) have implicated RNA synthesis among the macromolecular syntheses necessary for recovery of a high mutant yield, and GALLANT and CASHEL (1967) have demonstrated that the incorporation of uracil and UMP into RNA of stringent cells of *Escherichia*

coli is strongly influenced by the presence of an amino acid pool, it is thus possible that the "broth effect" on mutation induction with both EMS-treated *Bacillus subtilis* and UV-treated *Escherichia coli* is due to an immediate stimulation of RNA synthesis by the amino acid pool of the broth with its metabolic consequences.

WITKIN (1963) has reported that single step reversions of multiauxotrophic strains of *Escherichia coli* demonstrate a medial-dependent MFD, and it also appears from the work of BRIDGES, DENNIS and MUNSON (1967 b) that only the suppressor classes of reversions to prototrophy of *Escherichia coli B/r WP2 try*$^-$ show both "broth effect" and MFD. The nonsuppressor class, including presumably the true reversions, shows no such effects. Experiments are in progress to ascertain whether the response to posttreatments of true reversions induced by EMS-treatment of *Bacillus subtilis* strains also differs from that of the super-suppressor mutants described in this communication.

Liquid Media

The behaviour of EMS-treated *Bacillus subtilis* in liquid media differs in two ways from that on solid media:

1. In contrast to the stabilisation of a high induced mutation yield by broth in the selective agar, broth in liquid media had no such effect. Fig. 3 shows that the MFD in liquid minimal salts + glucose medium was only fractionally reduced by the addition of broth, together with tryptophan and uracil. Raising the broth concentration to 50% v/v produced only a slight sparing in MFD.

2. In contrast to the full MFD noted on solid medium in the presence of excess quantities of the four growth requirements of the strain (Table 2), addition of only two of these requirements to the liquid minimal salts + glucose medium used for intermediate incubation (leucine and uracil, or more complex mixtures containing added leucine and uracil) produced a marked sparing of MFD as shown in Fig. 3. It is unlikely that synthetic processes are responsible for this MFD-sparing by leucine and uracil as it occurred even when the cells had been previously starved of the other two growth requirements, histidine and tryptophan. Not only did leucine and uracil addition reduce the MFD of mutants recoverable on broth-supplemented selective agar, it also abolished the MFD of mutants recoverable on selective agar with traces of the previously required growth factors.

In so far as it is possible, it has been excluded that the absence of a "broth effect" in liquid media is due to such factors as the participation of a soluble agar component in mutation fixation or an osmotic instability of treated cells in liquid media. It is not possible to test for a selective death peculiar to the mutant fraction of the population in liquid media, however a probable explanation of these phenomena lies in a "washing-out" of residual mutagen or a recovery during liquid holding.

Residual mutagen or mutagenic intermediates will lead to different effects, dependent on whether nor not their action continues after plating. If it ceases to be mutagenic on plating treated cells, then the highest possible mutation frequencies are not seen on immediate plating. Higher mutation frequencies will be obtained when the treated and washed cells are kept in a pellet for some time

Table 3. *The effect of storage of treated cells as centrifuged pellets on mutation frequency and viability*

Posttreatment. Experiment		Viable cells per ml $\times 10^8$		Mutants scored/ml		Mutants/10^7 survivors	
		SA + broth	SA + traces of histidine and leucine	SA + broth	SA + traces of histidine and leucine	SA broth	SA + traces of histidine and leucine
Immediate resuspension and plating	A	1.93	1.98	4,390	1,020	228	52
	B	1.20	1.29	1.610	480	134	37
Held as a pellet for 2 hours at 0° C resuspended and plated	A	1.66	1.47	3,570	990	215	66
	B	1.23	0.92	1,590	520	129	57
Held as a pellet for 2 hours at 37° C, resuspended and plated	A	0.92	0.74	2,120	580	231	78
	B	0.46	0.42	670	220	146	52

Each figure was derived from the mean of three plates.

SA = selective agar = minimal salts + glucose agar + 30 μg/ml each of uracil and tryptophan. SA + broth = selective agar + 1.5% v/v nutrient broth. SA + traces of histidine and leucine = selective agar + 1.5 μg/ml each of histidine and leucine.

before plating as the residual mutagen now has a longer period in which to be mutagenic before its inhibition on plating. The "after-effects" of diepoxybutane and ethylene oxide in conidia of *Neurospora crassa* have been explained in this way (KØLMARK and KILBEY, 1962; KILBEY and KØLMARK, 1968), but no similar "after-effect" was obtained in the present experiments. This rules out residual mutagen that acts only before plating. On the other hand, the residual mutagen may continue to act even in the plating medium, when the mutation frequency observed on immediate plating will be the highest obtainable and will not be increased by keeping the cells in a pellet before plating. It may however be reduced by prolonged shaking in suspension. It is shown in Table 3 that the findings of the present experiments agree well with these predictions, and the possibility that the loss of mutations during posttreatment agitation in liquid may be due to "washing-out" of residual mutagen cannot be excluded. It is not a very likely one however, as it is difficult to see how leucine and uracil addition should specifically interfere with "washing-out". Moreover, in non-lysogenic strains of *Escherichia coli K 12*, mutation frequency after treatment with EMS was not changed by posttreatment agitation in minimal medium or buffer (SCHWARTZ, 1963; VERLY, BARBASON, DUSART and PETISPAS-DEWANDRE, 1967). Such species differences between bacteria are more readily understood in terms of differences between cellular steps in the mutation process than simply as differences in permeability to traces of trapped mutagen or its mutagenic intermediates.

As an alternative explanation, one may consider a liquid holding recovery, as the decline of EMS-induced mutation frequency in *Bacillus subtilis* is analogous in its independence of medial composition and dependence on temperature (Fig. 4) to that described by ROBERTS and ALDOUS (1949) for the restoration

of viability of UV-irradiated cells of an *Escherichia coli* strain. Repair mechanisms have been implicated in the reduction of lethality of *Escherichia coli* strains in liquid media (CASTELLANI, JAGGER and SETLOW, 1964; HARM, 1966), perhaps during a division delay induced by the liquid treatment (JAGGER, WISE and STAFFORD, 1964). A similar phenomenon has been described by ZETTERBERG (1966) for mutations induced by UV-irradiation of *Ophiostoma* conidia where a reduction in mutation frequency occurred only in liquid media. The assumption that a liquid holding recovery after EMS-treatment occurs in *Bacillus subtilis* is open to test by genotypic and phenotypic interference with the repair mechanisms presumably involved, as has been done for strains of *Escherichia coli* by HARM (1966). Such tests might also show whether there are any steps in common between MFD and the additional decline of mutation frequency in liquid media.

Of particular interest is the sparing of MFD in liquid media by the addition of leucine and uracil. If this is due to a specific interference of these two nutrilites with a liquid holding recovery mechanism, then leucine and uracil might also reduce any liquid holding recovery of other mutations in *Bacillus subtilis*. This possibility will be tested.

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ANALYSIS OF AN APPARENT CASE OF "GENE-CONTROLLED MUTATIONAL STABILITY": THE AUXOTROPHIC PREEMPTION OF A SPECIFIC GROWTH REQUIREMENT

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SUMMARY

The majority of spontaneous his$^+$ revertants of a his$^-$ threo$^+$ strain of *Bacillus subtilis* arose on the plates. Few, if any, such revertants arose when a his$^-$ threo$^-$ strain was plated on the same selective medium with an additional 30 μg/ml of threonine. An inhibitory role of this supplement on the formation of his$^+$ revertants was excluded. The inhibitory effect on spontaneous his$^+$ reversion thus seemed to be due to the threonineless genetic background. Further analysis, however, revealed that it was due to an interaction, not previously reported, between genetic background and medial factors. It was shown that concentrations of threonine which allowed normal growth of the his$^-$ threo$^-$ strain were not sufficient for the growth of his$^+$ reversions, most of which arose late on the plates. Addition of excess threonine allowed his$^+$ reversions to develop. It will be necessary to consider the possibility of such an auxotrophic preemption of medial growth factors in mutation experiments with multiple auxotrophic strains.

INTRODUCTION

In studies of bacterial mutation from auxotrophy to prototrophy, mutants are routinely selected on an agar medium from which a growth factor has been omitted. Frequently incorporated into the selective agar are trace quantities of the previously required growth factor, or of a richer mixture of amino acids, such as nutrient broth, containing the growth factor. With traces of growth factor alone, expression of induced mutants is enhanced[5,6,8,27] and residual divisions may occur on the plates[2,3,9,16], often with concomitantly greater yields of spontaneous mutations from the increased cell population[2,3,9]. Traces of nutrient broth show a similar effect on spontaneous mutation[17], and, in addition, stimulate the fixation of induced mutations[5,6,8,14,15,19,20,24,25]. Cases are on record where a selection against mutants

* This paper should have been incorporated in the special issue, dedicated to Professor CHARLOTTE AUERBACH, to honour her on the occasion of her official retirement, but due to unforeseen circumstances it was received after the deadline.

has occurred due to the exhaustion of the medial energy source by background growth of non-mutated cells[11,12,22,23].

The discovery[2,3] of a "gene-controlled mutational stability" in *Escherichia coli* followed the observation that the occurrence of spontaneous mutants during background growth of a try$^-$ auxotroph was prevented by the addition of another auxotrophy, for adenine; the second mutation preventing the appearance of spontaneous or induced try$^+$ mutants without contribution from medial factors. This communication reports a phenomenon of selection against revertants of a strain of *Bacillus subtilis*, at first thought similar to the "gene-controlled mutational stability" of *E. coli*. On subsequent examination, it proved to be due to the preemption by background growth of an amino acid required both by mutant and non-mutant cells.

MATERIALS AND METHODS

Bacterial strains

Strains of *B. subtilis* were constructed with combinations of a threo marker, the his-A1 marker, and their wild-type and revertant alleles as shown below:

his-A1$^-$ threo$^+$ → his-A1$^+$ threo$^+$ by spontaneous mutation
↓ → his-A1$^+$ threo$^+$ by transformation
his-A1$^-$ threo$^-$ → his-A1$^+$ threo$^-$ by spontaneous mutation
→ his-A1$^+$ threo$^-$ by transformation

The parent strain was a quadruple auxotroph, leu$^-$ his-A1$^-$ ura-1$^-$ try-2$^-$, previously described[8], and all strains shown in the diagram thus also require leucine, uracil, and tryptophan. The threo$^-$ marker was isolated as one of a few auxotrophies from a wild type strain following attempted 2-aminopurine mutagenesis, and it may therefore be of spontaneous origin. It was introduced to the quadruple auxotroph by transformation and recovered after penicillin selection. Transformation of his$^-$ strains to his$^+$ was performed with wild-type DNA. The threo$^+$ and threo$^-$ strains with independent mutations to his$^+$ still require leucine: these mutations are therefore only to histidine prototrophy and are not leu$^-$ his-A1$^-$ su$^+_{\text{leu,his-A1}}$ mutants as previously reported from the quadruple auxotroph[8]. Unfortunately, mutations to his$^+$ leu$^-$ arise only spontaneously in this strain and their induction by physical or chemical agents cannot be studied.

Reconstruction experiments cited in RESULTS were performed with his$^+$ threo$^+$ or his$^+$ threo$^-$ strains obtained by transformation with wild-type DNA. The corresponding his$^+$ strains obtained by spontaneous mutation from his$^-$ strains behaved in identical fashion in such experiments.

Media and preparation of cultures have been described elsewhere[8]. Briefly, spontaneously occurring histidine revertants were isolated on a selective minimal agar with 0.5% glucose and 30 μg/ml each of leucine, uracil, tryptophan—and threonine if required—with the addition of small quantities of histidine (usually 1 μg/ml) or "Oxoid" Nutrient Broth (usually 1.0–1.5% v/v). Variations of these supplement concentrations used are shown where appropriate in the text. (L-Amino acids and uracil were supplied by British Drug Houses Ltd.)

For estimation of mutants, 0.1-ml aliquots of cell suspension were spread on replicate plates of selective medium with a bent glass rod, and the plates incubated at 37° for 4 days when colony numbers were scored. Total viable counts were scored

after overnight incubation on Nutrient Broth Agar after plating at greater dilution. Transformation was performed by the technique of ANAGNOSTOPOULOS AND SPIZIZEN[1] using crude DNA preparations[13].

RESULTS

When 10^7–10^8 cells of a his$^-$ threo$^+$ mutant of *B. subtilis* were plated on selective agar without histidine, only a few his$^+$ revertant colonies were recovered. Addition of 1 μg/ml of histidine to the selective agar yielded a great many his$^+$ revertants over a wide range of initial plating densities. It is thus apparent that the great majority of these his$^+$ revertants arose during residual growth of the inoculum on trace-histidine-supplemented medium. This phenomenon is illustrated in Table I where it will be seen that approximately the same number of revertants was obtained per plate whether $4.9 \cdot 10^7$ or $4.9 \cdot 10^6$ cells were plated. These his$^+$ revertants grew well in the total absence of histidine after isolation. Similar phenomena have been described for a try$^-$ strain of *E. coli* B/r (WP. 2)[2,3,9].

TABLE I

NUMBER OF SPONTANEOUS his$^+$ REVERTANTS DERIVED FROM his$^-$ threo$^+$ AND his$^-$ threo$^-$ STRAINS IN A TYPICAL EXPERIMENT

Strain	*Number of cells inoculated per plate*	*his$^+$ mutants (mean of 10 plates) arising on selective medium + 1 μg/ml histidine*	
		Without added threonine	*+ 30 μg/ml threonine*
his$^-$ threo$^+$	$4.9 \cdot 10^7$	128	151
	$4.9 \cdot 10^6$	124	114
his$^-$ threo$^-$	$7.0 \cdot 10^7$	—	0
	$7.0 \cdot 10^6$	—	0

In contrast, his$^+$ revertants were not obtained in similar fashion from a his$^-$ threo$^-$ derivative of the his$^-$ threo$^+$ strain, to which an independently isolated threo$^-$ marker had been added by transformation. His$^+$ revertants were rarely isolated from this his$^-$ threo$^-$ strain although his$^-$ threo$^+$ clones derived from it by transformation with wild-type DNA yielded his$^+$ revertants in a manner similar to the original his$^-$ threo$^+$ parent.

As the selective medium for isolation of his$^+$ revertants from the his$^-$ threo$^-$ strain obligatorily contained additional threonine, test was made of the ability of threonine in the medium to inhibit the production of his$^+$ revertants by the his$^-$ threo$^+$ strain. An effect of medial supplements on the production of mutants has been previously reported[4], however it is shown in Table I that the his$^-$ threo$^+$ strain produced his$^+$ revertants in the presence or absence of gratuitous threonine. The total lack or greatly reduced number of his$^+$ revertants from the his$^-$ threo$^-$ strain cannot therefore be ascribed to the presence of threonine in the plating medium but to the presence in the cells of the threonine requirement. Thus far the system has much in common with a case of "gene-controlled mutational stability" reported for a try$^-$ mutant of *E. coli*[2,3] where the introduction of an additional ad$^-$ requirement stabilised the normally revertible try$^-$ marker to spontaneous (and in that instance, induced) mutation.

In contrast to the *E. coli* system however, revertants were detected in small

numbers in some experiments with the his⁻ threo⁻ strain. When the trace supplementation of histidine in the selective medium was replaced by a small quantity of nutrient broth (1.0–1.5% v/v), variable numbers of his⁺ revertants per plate were obtained from the his⁻ threo⁻ strain. Variable numbers of mutants on parallel plates have been previously reported[11] in a study of the effect of selection against revertants attributable to the exhaustion of the medial energy source. These facts argue against a complete mutational stability imposed on the his⁻ marker by the threonine requirement and suggest a selection against prototrophs arising on the plates.

Before attempting a more complex analysis, examination was first made to determine whether the genotype of isolated his⁺ revertants with respect to threonine affected selection against them in reconstruction experiments. Plates of selective medium containing 30 μg threonine and 1 μg histidine per ml were spread with aliquots of his⁻ threo⁻ cells as background. Immediately, or after 1, 2 or 3 days of incubation of this background, small numbers of either his⁺ threo⁺ or his⁺ threo⁻ cells were spread on to agar of similar composition to estimate their numbers, and upon agar preseeded with his⁻ threo⁻ background. All plates were then further incubated to allow any growth of added histidine prototrophs. Results given in Table II show clearly that whereas his⁺ threo⁺ and his⁺ threo⁻ cells grew if plated at the same time as a his⁻ threo⁻ background (0 h, columns A, B, D, E), only his⁺ threo⁺ grew if added thereafter (columns A, D), his⁺ threo⁻ cells being inhibited (columns B, E). It is evident that the discrimination against his⁺ threo⁻ cells by a his⁻ threo⁻ background is exerted, not immediately, but during and after the first 24 h of incubation. It is thus probable that the only his⁺ revertants to be recovered from a his⁻ threo⁻ inoculum will be those arising before discrimination becomes effective, thereby accounting for the small number of such revertants (if any) found on selective medium with 30 μg threonine and 1 μg histidine per ml. Inhibition of growth of added his⁺ threo⁻, but not of his⁺ threo⁺ cells, suggests that discrimination is due to the exhaustion of medial threonine by the his⁻ threo⁻ background.

TABLE II

RECONSTRUCTION EXPERIMENT: THE ABILITY OF SELECTIVE MEDIUM + 30 μg/ml THREONINE + 1 μg/ml HISTIDINE TO SUPPORT THE GROWTH OF A NUMBER OF his⁺ threo⁻ OR his⁺ threo⁺ CELLS ADDED AT VARIOUS TIMES AFTER THE INOCULATION OF $1.1 \cdot 10^7$ his⁻ threo⁻ CELLS PER PLATE

Time (h) of inoculation of histidine prototrophs after seeding of plates with his⁻ threo⁻ background and incubation at 37°	*his⁺ colonies per mean of 5 plates*				
	A *Number of his⁺ threo⁺ cells added*[a]	*B* *Number of his⁺ threo⁻ cells added*[a]	*C* *his⁻ threo⁻ background only*	*D* *his⁻ threo⁻ background + added his⁺ threo⁺ cells*	*E* *his⁻ threo⁻ background + added his⁺ threo⁻ cells*
0	152	125	3	126	115
24	231	211	0	255	0
48	241	76	0	243	0
72	395	256	0	454	0

[a] Scored on selective medium + 30 μg threonine and 1 μg histidine per ml without added background.

Directly comparable experiments with a his⁻ threo⁺ background could not be performed as large numbers of his⁺ revertants are formed by this strain, and these would be respread on addition of the daily ration of histidine prototrophs. A technique

was therefore devised in which the background of his⁻ threo⁻ or his⁻ threo⁺ cells were incorporated *in* selective agar with 30 μg threonine and 1 μg histidine per ml and covered when solidified with a thin sterile layer of such agar on which the reconstruction prototrophs were spread at daily intervals. Discrimination against histidine prototrophs takes longer to be manifested when the background was *in* rather than *on* the agar, but where comparable, the pattern of inhibition was the same. These experiments showed that his⁺ threo⁻ cells, and not his⁺ threo⁺, were inhibited by a his⁻ threo⁻, but not by a his⁻ threo⁺, background. This finding strengthens the suggestion that discrimination is due to an exhaustion of medial threonine.

To determine the influence of the extent of background growth on selection against his⁺ revertants, experiments were then performed in which the quantity of histidine and/or nutrient broth allowing residual growth on the plates was varied. The result of such an experiment is shown in Table III. It is evident that (a) the number of his⁺ revertants recovered from a his⁻ threo⁺ inoculum increased with greater medial supplementation, whether of histidine and/or broth, irrespective of a tenfold reduction in plating density. A similar increase of revertants with increasing broth supplementation has been shown for a met⁻ strain of *E. coli* K12 (ref. 17).

TABLE III

EFFECT OF HISTIDINE AND/OR NUTRIENT BROTH CONCENTRATION ON THE YIELD OF his⁺ REVERTANTS FROM his⁻ threo⁺ AND his⁻ threo⁻ STRAINS ON SELECTIVE AGAR + 30 μg/ml EACH OF LEUCINE, URACIL, TRYPTOPHAN, AND THREONINE

Each figure represents the mean of 3 plates.

Concentrations of added supplement		*Strain and inoculum of cells added per plate: yield of his⁺ revertants*			
Nutrient broth (% v/v)	*Histidine (μg/ml)*	*his⁻ threo⁺*		*his⁻ threo⁻*	
		$2.2 \cdot 10^7$	$2.2 \cdot 10^6$	$2.9 \cdot 10^7$	$2.9 \cdot 10^6$
—	—	14	0	22	2
0.1	—	20	5	35	6
0.25	—	21	9	45	11
0.5	—	70	26	32	30
1.0	—	74	77	24[a]	80[a]
1.5	—	99	166	19[a]	51[a]
—	0.1	43	15	44	25
—	0.25	48	37	59	49
—	0.5	80	67	38[a]	43[a]
—	1.0	151	121	5[a]	4[a]
—	1.5	99	84	9[a]	18[a]
0.5	0.5	101	124	13[a]	23[a]
1.0	0.5	160	132	2[a]	23[a]
0.5	1.0	91	136	0	5[a]
0.25	0.25	72	62	29[a]	25[a]

[a] Denotes colonies very much reduced in size.

(b) A his⁻ threo⁻ inoculum allowed recovery of large numbers of his⁺ revertants only on media of low supplementation; as background growth was increased by heavier supplementation with histidine and/or broth, a selection against his⁺ revertants became evident. This selection was more marked on histidine-supplemented agar than on agar supplemented with broth, and in the latter case was influenced by plating density. His⁺ revertants recovered from a his⁻ threo⁻ inoculum formed

markedly smaller colonies when medial supplementation reached levels of 1.0% v/v broth or 0.5 μg/ml histidine.

To examine the suggested exhaustion of medial threonine, inocula of his$^-$ threo$^-$ cells were incubated on selective media with 1 μg/ml histidine and various concentrations of threonine, as shown in Table IV. An increase in medial threonine

TABLE IV

EFFECT ON YIELD OF his$^+$ REVERTANTS OF VARYING CONCENTRATIONS OF THREONINE OR LEUCINE
Unless stated, selective medium contained 30 μg/ml each of leucine, uracil, tryptophan, and threonine, and 1 μg/ml histidine. Approx. 10^7 cells of his$^-$ threo$^-$ strain inoculated per plate.

Concentration of amino acid in selective medium (μg/ml)		*his$^+$ revertants per mean of 10 plates Expt. No.*				
Threonine	*Leucine*	*1*	*2*	*3*	*4*	*5*
30	30	1	3	9	4	5
60	30	12	38	39	54	35
100	30	24	35	71	73	77
300	30	166	102	142	54	69
30	60	1	1	0	—	—
30	100	1	2	3	—	—
30	300	1	3	2	—	—

concentration allowed recovery of numbers of his$^+$ revertants to a degree comparable with that normally recovered from a his$^-$ threo$^+$ inoculum. This illustrates convincingly that the his$^-$ threo$^-$ strain exerts its discrimination against his$^+$ revertants by exhaustion of medial threonine. Yet in growing cultures the threonine concentration of 30 μg/ml routinely used was sufficient to allow a growth rate and final cell density of the his$^-$ threo$^-$ strain comparable to its his$^-$ threo$^+$ parent, and to allow wild-type growth rate and final cell density to the original single threo$^-$ auxotroph.

In Table IV, it is also shown that an alternative increase in leucine concentration does not allow comparable recovery of greater numbers of his$^+$ revertants. Further, addition of leucine, uracil, tryptophan, and threonine at 30 μg/ml or singly or in combination at 100 μg/ml yielded numbers of his$^+$ revertants dependent on the threonine concentration. It is evident therefore that threonine deprivation is the only factor in discrimination against his$^+$ revertants by a his$^-$ threo$^-$ background, as suggested by results of reconstruction experiments cited in Table II and in the text.

It is possible that the difference observed in yield of his$^+$ revertants from the his$^-$ threo$^-$ strain on media with various concentrations of threonine (Table IV) was not caused by the permission of growth of revertants by suitable threonine concentrations but by an effect of such threonine on the amount of residual growth of the inoculum. Such a threonine-dependent regulation of the residual growth on selective medium with 1 μg/ml histidine could influence the observed numbers of spontaneous his$^+$ revertants in a similar manner. Resuspension and counting experiments have shown however that the extent and rate of background growth of a his$^-$ threo$^-$ inoculum on selective medium+1 μg histidine and 30 or 100 μg threonine per ml was the same. It was concluded that histidine at 1 μg/ml is the only restricting factor for a his$^-$ threo$^-$ inoculum, 30 μg/ml threonine allowing full background growth but not growth of revertants.

Further examination of the specificity of discrimination against revertants has

shown that such discrimination occurred only for his$^+$ revertants from a his$^-$ threo$^-$ strain. A his$^-$ threo$^+$ strain yielded revertants in the presence or absence of gratuitous threonine (Table I). Spontaneous threo$^+$ revertants on the other hand were produced on selective medium with 30 μg histidine and 1 μg threonine per ml by a his$^-$ threo$^-$ strain, and by a his$^+$ threo$^-$ strain in the presence or absence of gratuitous histidine.

DISCUSSION

An assumption implicit in mutation experiments is that, after allowance is made for repair mechanisms, the observed mutation frequencies represent the responses of the studied alleles to spontaneous or induced mutagenesis. Influences of genetic background and composition of the plating medium are often disregarded. There are however sufficient examples in the literature of the modification of mutation frequencies by such influences to warrant caution.

For example, there may be (a) an indirect effect of growth medium; the background of non-mutated cells may inhibit mutant growth either by exhaustion of medial energy sources[11,12,22,23] or by the production of inhibitory substances[22,23]; (b) a direct effect of growth medium; nutrients, whether gratuitously added or required by the strain under study, may inhibit mutant growth. Mutation frequency from ad$^-$ to ad$^+$ in a strain of *Schizosaccharomyces pombe* was much reduced by the addition of gratuitous methionine to the monoauxotroph, also in an ad$^-$ met$^-$ diauxotroph where methionine was an obligatory medial component[4]. With the diauxotroph therefore, methionine mimicked an apparent genetic effect of the met$^-$ mutation. Interestingly, the degree of methionine suppression of mutation frequency was mutagen-specific, being much more pronounced for ultraviolet light than for nitrous acid; (c) a true effect of genetic background; this has been demonstrated for a strain of *E. coli* where an effect of medial components has been specifically excluded[2,3]. Try$^+$ mutants arose during residual growth on mutation assay plates, and higher mutation frequencies could be induced by a number of mutagens. The addition of a specific adenine requirement totally prevented recovery of any try$^+$ mutants although ad$^+$ mutants were recovered. Other presumed genetic background effects have been reported for *E. coli*[10], *Aspergillus nidulans*[18]; and *S. pombe*[7] although plating medium effects have not been rigorously excluded in all these instances.

By comparison, the present report describes an interaction between genetic background and medial factors with resultant discrimination against revertants. At concentrations of medial threonine which allow normal growth of the his$^-$ parent, his$^+$ revertants were selected against in a threo$^-$, but not in a threo$^+$, background. This has been demonstrated in two ways. Firstly, reconstruction experiments (*e.g.* Table II) showed that his$^+$ revertants were inhibited by a his$^-$ threo$^-$ background only if they also were threo$^-$ and only when the background had been allowed some growth. His$^+$ threo$^-$ revertants were inhibited if added 24 h later than the background but not when added at the same time, suggesting that threonine could be exhausted from the medium by background growth. The amount of such background growth has been shown to be dependent on the 1 μg/ml of histidine present, not on the concentration of threonine present. Secondly, his$^+$ revertants arose in greater numbers as the medial content of threonine was increased (Table IV), although the amount of background growth was limited by a constant 1 μg/ml of histidine. Threonine

was the only medial component to be preempted by the auxotrophic background which does not therefore render corresponding quantities of leucine, uracil, or tryptophan unavailable to revertants arising on the plates. There was no evidence of a reciprocal discrimination against threo$^+$ revertants. It would be of interest to determine whether mutants of other genes arising on plates are discriminated against under the same conditions as his$^+$ revertants. The mechanism of threonine exhaustion has not been examined and must await enzymatic characterization of the threo$^-$ mutation for possible explanation.

In mutation experiments with multiple auxotrophs therefore, the auxotrophic preemption of growth factor(s) required both by mutant and nonmutant cells must be considered for possible effects on observed mutation frequency. In the *E. coli* system already cited, a similar effect on try$^+$ revertants by adenine deprivation has been ruled out (V. L. CHOPRA, personal communication). Mutants likely to be discriminated against are those arising late on the plates of selective media. These may be of different types: (a) spontaneous mutants, some of which arise only on the plates. This can be inferred when their number per plate is largely independent of the size of the inoculum. The reason for this is that the final population size achieved on the plates may depend very little on the original inoculum size, but on the amount of residual growth permitted to this inoculum. The final population size determines the number of spontaneous mutants observed[2,3,9] unless a discrimination against mutants is exerted by genetic or medial influences, or, as shown in this report, by an interaction of these factors (Table III); (b) induced mutants arising by the delayed action of mutagenic treatments; (c) mutants induced by massive doses of mutagen which simultaneously cause great delay to the growth and division of mutants[21]; and (d) suppressor or partial revertants with growth rates much reduced from those of their parent auxotrophs and wild-type equivalents.

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